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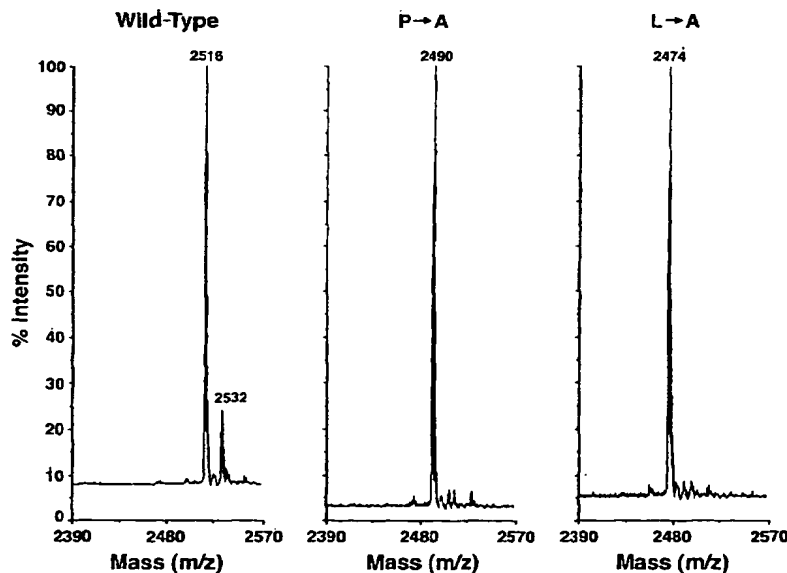
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(54) Title: PHARMACEUTICALS AND METHODS FOR TREATING HYPOXIA AND SCREENING METHODS THEREFOR



(57) Abstract: Light-generating fusion proteins having a ligand binding site and a light-generating polypeptide moiety and their use as diagnostics, in drug screening and discovery, and as therapeutics, are disclosed. The light-generating fusion protein has a feature where the bioluminescence of the polypeptide moiety changes upon binding of a ligand at the ligand binding site. The ligand may be, for example, an enzyme present in an environment only under certain conditions, e.g., ubiquitin ligase in a hypoxic state, such that the light-generating fusion protein is "turned on" only under such conditions.

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PHARMACEUTICALS AND METHODS FOR TREATING HYPOXIA AND SCREENING METHODS THEREFOR

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with U.S. government support. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

How cells sense changes in ambient oxygen is a central problem in biology. In mammalian cells, lack of oxygen, or hypoxia, leads to the stabilization of a sequence-specific DNA-binding transcription factor called HIF (hypoxia-inducible factor), which transcriptionally activates a variety of genes linked to processes such as angiogenesis and glucose metabolism.

Tissue ischemia is a major cause of morbidity and mortality. Ischemia can result from chronic hypoxia brought on by lack of blood supply to the tissue occurring from, for example, stroke, deep vein thrombosis, pulmonary embolus, and renal failure. Ischemic tissue is also found in tumors.

HIF binds to DNA as a heterodimer consisting of an alpha subunit and a beta subunit (also called aryl hydrocarbon receptor nuclear translocator or ARNT). The alpha subunit is rapidly polyubiquitinated and degraded in the presence of oxygen whereas the beta subunit is constitutively stable. von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome characterized by the development of highly vascular tumors that overproduce hypoxia-inducible mRNAs such as vascular endothelial growth factor (VEGF). The product of the VHL tumor suppressor gene, pVHL, is a component of multiprotein complex that contains elongin B, elongin C, Cul2, and Rbx1. This complex bears structural and functional similarity to SCF (Skp1/Cdc53 or Cullin/F-box) ubiquitin ligases. In the presence of oxygen pVHL binds directly to HIF α subunits and targets them for polyubiquitination and destruction. Cells lacking functional pVHL can not degrade HIF and thus overproduce mRNAs encoded by HIF-target genes.

Progress has also been made in recent years towards understanding the molecular mechanisms in control of cell proliferation. Aberrant cell proliferation is a hallmark event in the onset and progression of diseases such as cancer. Progression through the mammalian cell-cycle is linked to the orchestrated appearance and destruction of cyclins.

Different cyclins are associated with different cell-cycle transitions. For example, cyclin E is active in late G1 and early S-phase, cyclin A is active in S-phase, and cyclin B is active in mitosis. Cyclins bind to cyclin-dependent kinases (cdks). In this context, cyclins activate the catalytic activity of their partner cdk(s) and also play roles in substrate recognition.

5 Some transcriptional regulatory proteins, such as the pRB homologs p107 and p130, the E2F family members E2F1, E2F2, and E2F3, the transcriptional coactivator p300, and NPAT (nuclear protein mapped to the AT locus) form stable complexes with cyclin A/cdk2 and/or cyclin E/cdk2. All of these proteins bind directly or indirectly to DNA. Thus, such complexes might serve as vehicles for increasing the concentration of cyclin A/cdk2 or
10 cyclin E/cdk2 at certain sites within the genome. As such, cyclin A/cdk2 and cyclin E/cdk2 might play relatively direct roles in processes such as transcription and DNA replication. These two processes are fundamental in normal cell proliferation and are perturbed during aberrant cell proliferation, such as in cancer.

SUMMARY OF THE INVENTION

15 The present invention relates to, in part, the discovery that modulation of the activity of HIF, which is regulated via its hydroxylation status by prolyl hydroxylase, may be used to control or mediate disease conditions (e.g., *in vivo*) such as hypoxia or cell-proliferating disorders like cancer, through the use of therapeutic compounds which modulate prolyl hydroxylase or the hydroxylation or non-hydroxylation of the segment of HIF responsible
20 for its degradation.

 As such, the invention relates in one aspect to methods of treating or preventing hypoxic or ischemic related disorders in a subject, wherein therapeutic compounds which modulate prolyl hydroxylation of HIF are administered to a subject, such that the hypoxic or ischemic related disorder is treated. The compounds may increase or decrease prolyl
25 hydroxylation of HIF.

 Alternately, HIF turnover in a subject may be regulated in a subject by administering to a subject in need thereof a therapeutic compound which modulates (increases or decreases) prolyl hydroxylation of HIF, such that HIF turnover is regulated.

 HIF-related disorders, e.g., myocardial infarction, stroke, cancer, or diabetes, may
30 be treated by administering to a subject in need thereof a compound which modulates prolyl hydroxylation of HIF, such that the disorder is prevented, reversed or stabilized.

In accordance with the invention, prolyl hydroxylase modulators may be identified using several ways. In one embodiment, a prolyl hydroxylase, a putative prolyl hydroxylase modulator and a light-generating fusion protein comprising an HIF α polypeptide moiety having binding character for prolyl hydroxylase and a light-generating polypeptide moiety, wherein the light generation of the light-generating polypeptide moiety changes upon binding of a prolyl hydroxylase to the HIF α polypeptide moiety, are contacted under conditions favorable for binding of a prolyl hydroxylase to a HIF α to form a test sample; and the ability of the putative prolyl hydroxylase modulator to modulate prolyl hydroxylase is determined by measuring the light generated in the test sample.

Assays for prolyl hydroxylase modulators are included in the invention, wherein a prolyl hydroxylase, a HIF1 α polypeptide moiety and a putative modulator compound are brought into contact under conditions where prolyl hydroxylase, in the absence of modulator, is capable of acting on the HIF1 α polypeptide moiety; and the degree of prolyl hydroxylase inhibition caused by the modulator compound is measured. The test sample, in an advantageous embodiment, may further comprise pVHL. Desirably the HIF1 α polypeptide moiety comprises the amino acid sequence Y-X₁-Leu-X₂-Pro_h-X₃-X₄-X₅-X₆-Y', wherein Pro_h is hydroxylated proline; X₁, X₂, X₄, X₅, and X₆ are independently Gly, Ala, Val, Leu, Ile, Pro, Met, Phe, or Trp; X₃ is Ser, Thr, or Tyr; and Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids. In further advantageous embodiments, the HIF1 α polypeptide moiety comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline; comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 402 is hydroxylated proline; or comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein either or both of residues 402 and 564 are hydroxylated proline. The HIF1 α polypeptide moiety may also comprise amino acids 555-575 of wild type HIF α , numbered in accordance with wild type HIF α .

The HIF1 α polypeptide moiety may alternately comprise a 4 to 12 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline; may comprise a 12 to 14 amino acid sequence corresponding to the

residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline; may comprise a 20 to 30 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline; or may comprise a 80 to 120 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.

The invention also includes HIF α polypeptides comprising amino acids 555-575 of wild type HIF α , numbered in accordance with wild type HIF α .

The invention relates in another embodiment to methods of treating or preventing a hypoxic or ischemic related disorders, such as myocardial infarction, stroke, cancer, or diabetes, in a subject, wherein compounds identified by the methods above decrease prolyl hydroxylase expression or activity, such that said hypoxic or ischemic related disorder is treated. Such compounds may be prolyl hydroxylase antibodies, or a nucleic acids (e.g., a prolyl hydroxylase anti-sense nucleic acid) that decreases the expression of a nucleic acid that encodes a prolyl hydroxylase polypeptide. Other hypoxic or ischemic related disorders may be treated by compounds of the invention, such as chronic events like deep vein thrombosis, pulmonary embolus, and renal failure.

The compounds of the invention find use in another aspect of the invention, treating cancer or increasing angiogenesis or vascularization, wherein compound of the invention, such as identified by the methods described herein, is administered to a subject in need thereof, so that prolyl hydroxylase expression or activity is decreased.

The invention relates in part to the use of light-generating fusion proteins (or cells expressing the light-generating fusion protein), wherein the light-generating fusion protein features a ligand binding site and a light-generating polypeptide moiety. The light-generating fusion protein ("LGP") has a feature where the light generation of the light-generating polypeptide moiety changes upon binding of a ligand at the ligand binding site. The ligand may be active in an environment only under certain conditions, e.g., in a hypoxic state, such that the light-generating fusion protein is "turned" on or off only under such conditions.

The light-generating fusion proteins of the invention may be used for screening for modulators of activity or latency of (or predisposition to) disorders such as hypoxia, cancer, diabetes, heart disease or stroke. For example, a test compound may be administered to a test animal at increased risk for such a disorder, wherein the test animal recombinantly
5 expresses a light-generating fusion protein, allowing for localization of the light-generating fusion protein, detecting the luminescence of the light-generating polypeptide moiety in the test animal after administering the test compound, and comparing the luminescence of the light-generating polypeptide moiety in the test animal with the luminescence of the light-generating polypeptide moiety in a control animal not administered the test compound. A
10 change in the activity of the light-generating polypeptide moiety in the test animal relative to the control animal indicates the test compound may be a modulator of, e.g., prolyl hydroxylase.

The effects of an anti-hypoxic compound *in vivo* may be determined in another embodiment, by administering to a subject, e.g., mammalian, a light-generating fusion
15 protein comprising an ubiquitin ligase binding site and a light-generating polypeptide moiety or a cell expressing the light-generating fusion protein, allowing for localization of the light-generating fusion protein or cell in hypoxic tissue in the subject; and measuring the luminescence of the localized light-generating fusion protein from the hypoxic tissue.

Methods in accordance with the invention are also provided for determining the
20 effects of an anti-cell proliferation compound under study in a mammalian subject, by administering to a test subject a light-generating fusion protein containing a cyclin/cdk binding site or a cell expressing the light-generating fusion protein of the invention, allowing for localization of the fusion protein or cell, measuring luminescence from the localized light-generating fusion protein; and imaging the localized light-generating fusion
25 protein, thereby determining the effects of the anti-cell proliferation compound.

The treatment of cell-proliferating disorders may be monitored by an embodiment of the invention, e.g., by administering to a subject an effective amount of a fusion protein comprising a HIF α polypeptide moiety having a binding affinity for prolyl hydroxylase, a suicide polypeptide moiety, and a light-generating polypeptide moiety, wherein the light
30 generation of the light-generating fusion protein changes upon binding of prolyl hydroxylase to the HIF α polypeptide moiety, such that the cell-proliferating disorder is treated, and monitoring the ability of the fusion protein to inhibit cell proliferation by measuring the light generated by the light-generating fusion protein. Alternately, treatment

of cell-proliferating disorders may be monitored by administering to a subject an effective amount of a light-generating fusion protein comprising a cyclin/cdk binding site, a suicide protein polypeptide moiety, and a light-generating polypeptide moiety, wherein the light generation of the light-generating fusion protein changes upon binding of a cyclin to the cyclin/cdk binding site, such that said cell-proliferating disorder is treated; and monitoring the ability of the fusion protein to inhibit cell proliferation by measuring the light generated by the light-generating fusion protein.

Other embodiments of the invention include antibodies specific for a protein complex comprising HIF1 α and pVHL are also detailed as within the present invention.

BRIEF DESCRIPTION OF THE DRAWING

FIG 1 is a schematic representation of different fusion proteins of the present invention.

FIG 2 is a second schematic representation of different fusion proteins of the invention.

FIG 3 shows pVHL binding to a modified form of HIF.

FIG 4 shows pVHL binding to a HIF1 α -derived peptide if Leu562 and Pro564 are intact.

FIG 5 shows ubiquitination and degradation of HIF linked to Leu562 and Pro 564.

FIG 6 depicts proline hydroxylation linked to pVHL-binding.

FIG 7 illustrates pVHL specifically recognizing HIF1 α with hydroxylated proline 564.

FIG 8 illustrates the production of TET α -cyclins A and E.

FIG 9 shows DNA bound cyclins A and E differentially affecting transcription.

FIG 10 illustrated transcriptional regulation by cyclins A and E dependent upon DNA binding.

FIG 11 depicts cyclin box is required for transcriptional repression by DNA bound cyclin A.

FIG 12 illustrates that transcriptional activation by cyclin E is linked to its ability to bind to cdk2 and interact with substrates.

FIG 13 shows that transcriptional activation by DNA bound cyclin E depends on cdk2 catalytic activity.

FIG 14 shows transcriptional effects mediated by cell-cycle dependent changes in endogenous cyclins E and A.

5 **FIGs 15-18** illustrate the results obtained in Example 2.

FIG 19 illustrates the wild type sequence of HIF1 α , Accession No. Q16665 (SEQ ID NO: 10).

FIG 20 illustrates kinase Cdk2, when bound to either cyclin E or cyclin A, can direct the phosphorylation of its substrates.

10 **DETAILED DESCRIPTION OF THE INVENTION**

Definitions

For convenience, certain terms used in the specification, examples, and appended claims are collected here.

15 “HIF α polypeptide moiety” includes all or part of the amino acid sequence of HIF1 α , HIF2 α , or HIF3 α .

20 “HIF1 α polypeptide moiety” includes all or part of the amino acid sequence of HIF1 α , e.g., SEQ ID NO: 10, the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein either or both of residues 402 and 564 are proline or hydroxylated proline, or an 80 to 120, 20 to 30, 12 to 14, or 4 to 12 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 402 and/or 564, inclusive, of HIF1 α , wherein residues 402 and/or 564 is proline or hydroxylated proline.

25 “Colinear effector site” includes regions of the light-generating polypeptide moiety that, when acted on by events subsequent to ligand binding, cause the light-generating polypeptide moiety to change its present light-emitting state (i.e., on or off). These regions making up the colinear effector site may do this by, e.g., conformational distortion, chemical modification, e.g., ubiquitination of a residue or residues in the colinear effector site, or by cleavage of a portion of all or part of the colinear effector site.

30 “Having binding character for prolyl hydroxylase” refers to a property making HIF α polypeptide moieties suitable for, e.g., screening methods of the invention and includes HIF

polypeptide sequences suitable or adapted for prolyl hydroxylase binding as well as native or wild-type HIF sequences to which pVHL has recognized and bound.

“Having binding character for ubiquitin ligase” refers to a property making HIF α polypeptide moieties suitable for, e.g., screening methods of the invention, e.g., including
5 native HIF polypeptide sequences having hydroxylated proline residues hydroxylated by prolyl hydroxylase, or, e.g., “HIF1 α polypeptide moieties” as defined herein.

“Localization” includes determining the particular region of the subject of an entity of interest, e.g., a tumor.

“Kemptide” includes a synthetic cAMP peptide substrate corresponding to part of
10 the phosphorylation site sequence in porcine liver pyruvate kinase. “Malantide” includes cAMP-dependent protein kinase and protein kinase C substrate in various tissues.

“Small molecule” includes compositions that have a molecular weight of less than about 5 kD and most preferably less than about 4 kD. Small molecules can be, e.g., nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic or
15 inorganic molecules. “Spread of infection” includes the spreading and colonization by a pathogen of host sites other than the initial infection site. The term can also include, however, growth in size and/or number of the pathogen at the initial infection site.

“Ligand” includes a molecule, a small molecule, a biomolecule, a drug, a peptide, a polypeptide, a protein, a protein complex, an antibody, a nucleic acid, or a cell.

20 “Ligand binding site” includes the location on the light-generating fusion protein to which a ligand binds, whereupon the light-generating polypeptide moiety is activated or inactivated as a direct or indirect consequence of ligand binding.. Binding to the ligand binding site may be direct or indirect, e.g., via protein dimerization in conjunction with other proteins, as described hereinbelow.

25 “Targeting moiety” includes moieties which allow the light-generating fusion protein of the invention to be selectively delivered to a target organ or organs. For example, if delivery of a therapeutic compound to the brain is desired, the carrier molecule may include a moiety capable of targeting the compound to the brain, by either active or passive transport. Illustratively, the carrier molecule may include a redox moiety, as described in,
30 for example, U.S. Pat. Nos. 4,540,564 and 5,389,623, both to Bodor. These patents disclose drugs linked to dihydropyridine moieties which can enter the brain, where they are oxidized to a charged pyridinium species which is trapped in the brain. Thus, compound

accumulates in the brain. Many targeting moieties are known, and include, for example, asialoglycoproteins (see, e.g. Wu, U.S. Patent No. 5,166,320) and other ligands which are transported into cells via receptor-mediated endocytosis. Targeting moieties may be covalently or non-covalently bound to a light-generating fusion protein. The targeting moiety may also be attached to a vector.

“Bioluminescent” molecules or moieties include luminescent substances which utilize chemical energy to produce light.

“Fluorescent” molecules or moieties include those which are luminescent via a single electronically excited state, which is of very short duration after removal of the source of radiation. The wavelength of the emitted fluorescence light is longer than that of the exciting illumination (Stokes’ Law), because part of the exciting light is converted into heat by the fluorescent molecule.

“Entities” include, without limitation, small molecules such as cyclic organic molecules; macromolecules such as proteins; polymers; polysaccharides; nucleic acids; particles, inert materials; organelles; microorganisms such as viruses, bacteria, yeast and fungi; cells, e.g., eukaryotic cells; embryos; prions; tumors; all types of pathogens and pathogenic substances; and particles such as beads and liposomes. In another aspect, entities may be all or some of the cells that constitute the mammalian subject being imaged, e.g., diseased or damaged tissue, or compounds or molecules produced by those cells, or by a condition under study. Entities for which the invention has particular utility include tumors, proliferating cells, pathogens, and cellular environments comprising hypoxic tissue.

“Infectious agents” include parasites, viruses, fungi, bacteria or prions.

“Promoter induction event” includes an event that results in the direct or indirect induction of a selected inducible promoter.

“Heterologous gene” includes a gene which has been transfected into a host organism. Typically, a heterologous gene refers to a gene that is not originally derived from the transfected or transformed cells’ genomic DNA.

“Opaque medium” includes a medium that is “traditionally” opaque, not necessarily absolutely opaque. Accordingly, an opaque medium includes a medium that is commonly considered to be neither transparent nor translucent, and includes items such as a wood board, and flesh and skin of a mammal.

“Light-generating” or “luminescent” includes the property of generating light through a chemical reaction or through the absorption of radiation, including phosphorescence, fluorescence, and bioluminescence.

5 “Light” includes electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

“Non-invasive” methods for detecting localization in a subject does not include largely invasive methods such as conventional surgery or biopsy.

10 “Light-generating fusion protein” includes proteins of the invention having a light-generating or luminescent portion, i.e., a light-generating polypeptide moiety and a ligand binding site. In general, when a ligand of interest binds to the ligand binding site of the light-generating fusion protein, the light-generating properties of the light-generating polypeptide moiety change, either going from “dark” to “light”, or vice versa.

15 “Light-generating polypeptide moiety” includes any protein known to those of ordinary skill in the art to provide a readily detectable source of light when present in stable form. Non-limiting examples include light-generating proteins described in U.S. Patent Nos. 5,683,888, 5,958,713, and 5,650,135, e.g., ferredoxin IV, green fluorescent protein, red fluorescent protein, yellow fluorescent protein, blue fluorescent protein, the luciferase family and the aequorin family. In a preferred embodiment, the light-generating polypeptide moiety is a protein such as green fluorescent protein, red fluorescent protein,
20 yellow fluorescent protein and blue fluorescent protein.

Tissue ischemia is a major cause of morbidity and mortality. In principle, drugs that stabilize HIF may augment angiogenesis and the adaptation of hypoxia. The activation of HIF by hypoxia is complex and involves protein stability, nuclear localization, DNA binding capability and transcriptional activation function. The discovery that proline
25 hydroxylatation governs HIF turnover in the presence of oxygen will facilitate the dissection of the mechanism underlying the various aspects of HIF regulation.

The invention also provides various methods of treating, *i.e.*, reducing, preventing or delaying the onset of HIF-1 related disorders, modulating angiogenesis or vascularization. Examples of HIF-1 mediated disorders include chronic and acute hypoxia or ischemia
30 related disorders such as tissue damage and scarring. Acute hypoxia or ischemia related disorders include for example myocardial infarction, stroke, cancer and diabetes such as

tissue damage and scarring. Chronic hypoxia or ischemia related disorders include for example, deep vein thrombosis, pulmonary embolus and renal failure.

In one aspect the invention provides methods of treating or preventing a hypoxic or ischemic related disorder or modulating angiogenesis or vascularization by administering to a subject a compound that decreases prolyl hydroxylase, expression or activity. Examples of prolyl hydroxylase includes human Egl-9 or homologs. (Epstein, et al *Cell* 107:43-54, 2001) The compound can be a prolyl hydroxylase antibody, a nucleic acid that decreases the expression of a nucleic acid that encodes a prolyl hydroxylase polypeptides such as a prolyl hydroxylase anti-sense nucleic acid or a compound identified by any of the methods of the invention. Preferably, the half life of HIF in the subject is increased in the presence of the compound as compared to the absence of the compound.

In a further aspect the invention includes a method of treating cancer in a subject by administering to the subject a compound that increases prolyl hydroxylase expression or activity. Preferably, the compound is a compound that has been identified by the methods of the invention.

In another aspect the invention provides a method for treating or preventing a hypoxia or ischemic related disorder in a subject by administering to a subject a compound that which modulates prolyl hydroxylation of HIF.

In still a further aspect the invention provides a method for treating or preventing a HIF related disorder by administering to a subject a compound that which modulates prolyl hydroxylation of HIF such that the HIF related disorder is prevented reversed or stabilized.

In yet another aspect the invention provides a method for regulating HIF turnover in a subject by administering to a subject a compound that which modulates prolyl hydroxylation.

By "modulates" is meant to increase or decrease the prolyl hydroxylation of HIF. Compounds that inhibits prolyl hydroxylation include several small molecule proline hydroxylase inhibitors which have been developed as antifibrotic agents.

The subject is preferably a mammal. The mammal can be, e.g., a human, non-human primate, mouse, rat, dog, cat, horse, or cow. In various aspects the subject include patients with coronary, cerebral, or peripheral arterial disease and patients with one or more non-healing wounds.

The invention further relates to methods of identifying or detecting prolyl hydroxylation, wherein the substrate peptide (or polypeptide) is contacted with pVHL, wherein the amount of pVHL bound reflects the degree of hydroxylation. In one embodiment, the peptide corresponds to HIF1a 555-575. The HIF peptide can be
5 immobilized (for example, on a nitrocellulose filter or at the bottom of a 96 well plate) or free in solution. Binding to pVHL can be monitored using a variety of standard methods familiar to those skilled in the art.

Modulation of prolyl hydroxylase has a variety of uses. Inhibiting prolyl hydroxylase may facilitate cell cycle progression and the production of a number of
10 proteins which promote angiogenesis and/or promote cellular survival or cellular function in hypoxia, a desirable outcome in the treatment of certain clinical conditions, particularly ischemic conditions such as coronary, cerebral and vascular insufficiency.

VHL used in assays of the invention may be any suitable mammalian VHL, particularly human VHL. Human VHL has been cloned and sources of the gene can be
15 readily identified by those of ordinary skill in the art. Its sequence is available as Genbank accession numbers AF010238 and L15409. Other mammalian VHLs are also available, such as murine VHL (accession number U12570) and rat (accession numbers U14746 and S80345). Non-mammalian homologues include the VHL-like protein of *C. elegans*, accession number F08G12. VHL gene sequences may also be obtained by routine cloning
20 techniques, for example by using all or part of the human VHL gene sequence as a probe to recover and to determine the sequence of the VHL gene in other species. A wide variety of techniques are available for this, for example, PCR amplification and cloning of the gene using a suitable source of mRNA (e.g., from an embryo or a liver cell), obtaining a cDNA library from a mammalian, vertebrate, invertebrate or fungal source, e.g., a cDNA library
25 from one of the above-mentioned sources, probing the library with a polynucleotide of the invention under stringent conditions, and recovering a cDNA encoding all or part of the VHL protein of that mammal. Suitable stringent conditions include hybridization on a solid support (filter) overnight incubation at 42°C in a solution containing 50% formamide, 5xSSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x
30 Denhardt's solution, 10% dextran sulfate and 20 µg/ml salmon sperm DNA, followed by washing in 0.03M sodium chloride and 0.03M sodium citrate (i.e. 0.2x SSC) at from about 50°C to about 60°C). Where a partial cDNA is obtained, the full length coding sequence may be determined by primer extension techniques.

It is not necessary to use the entire VHL protein (including their mutants and other variants). Fragments of the VHL may be used, provided such fragments retain the ability to interact with the target domain of the HIF α subunit. Fragments of VHL may be generated in any suitable way known to those of skill in the art. Suitable ways include, but are not limited to, recombinant expression of a fragment of the DNA encoding the VHL. Such fragments may be generated by taking DNA encoding the VHL, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out that portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Small fragments of the VHL (up to about 20 or 30 amino acids) may also be generated using peptide synthesis methods which are well known in the art. Generally fragments will be at least 40, preferably at least 50, 60, 70, 80 or 100 amino acids in size.

The HIF α subunit protein may be any human or other mammalian protein, or fragment thereof which has the ability to bind to a wild type full length VHL protein, such that the binding is able, in a normoxic cellular environment, to target the a subunit for destruction.

A number of HIF α subunit proteins have been cloned. These include HIF1 α , the sequence of which is available as Genbank accession number U22431, HIF2 α , available as Genbank accession number U81984 and HIF3 α , available as Genbank accession numbers AC007193 and AC079154. These are all human HIF α subunit proteins. HIF α subunit proteins from other species, including murine HIF1 α (accession numbers AF003695, US9496 and X95580), rat HIF1 α (accession number Y09507), murine HIF2 α (accession numbers U81983 and D89787) and murine HIF3 α (accession number AF060194). Other mammalian, vertebrate, invertebrate or fungal homologues may be obtained by techniques similar to those described above for obtaining VHL homologues.

Variants of the HIF α subunits may be used, such as synthetic variants which have at least 45% amino acid identity to a naturally occurring HIF α subunit (particularly a human HIF α subunit), preferably at least 50%, 60%, 70%, 80%, 90%, 95% or 98% identity.

Fragments of the HIF α subunit protein and its variants may be used, provided that the fragments retain the ability to interact with a wild-type VHL, preferably wild-type human VHL. Such fragments are desirably at least 20, preferably at least 40, 50, 75, 100,

200, 250 or 400 amino acids in size. Alternately, such fragments may be 12 to 14 amino acids in size, or as small as four amino acids. Most desirably such fragments include the region 555-575 found in human HIF1 α or its equivalent regions in other HIF α subunit proteins. Optionally the fragments also include one or more domains of the protein responsible for transactivation. Reference herein to a HIF α subunit protein includes the above mentioned mutants and fragments which are functionally able to bind VHL protein unless the context is explicitly to the contrary.

The invention further provides methods for testing putative inhibitor compounds for activity ("screening") in promoting HIF stabilization, e.g., contacting the compound, ischemic tissue, and the fusion protein of the invention under conditions appropriate to detect the fusion protein if the compound promotes HIF stabilization. The method (also referred to herein as a "screening assay") can be used for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) that promote HIF stabilization. The invention also includes compounds identified in the screening assays described herein, and pharmaceutical compositions for treatments as described herein.

In another embodiment, the invention relates to methods of identifying or detecting prolyl hydroxylation, wherein the substrate peptide or polypeptide is contacted with an antibody, wherein the amount of antibody bound reflects the degree of hydroxylation. In one embodiment, the peptide corresponds to HIF1 α 555-575. The HIF peptide may be immobilized (for example, on a nitrocellulose filter or at the bottom of a 96 well plate) or free in solution. Binding to the antibody can be monitored using a variety of standard methods familiar to those skilled in the art.

Yet another embodiment of the invention relates to methods of identifying or detecting prolyl hydroxylation wherein a polypeptide is translated in the presence of labeled, e.g., radioactive, proline and a prolyl hydroxylase, hydrolyzing the resulting labeled polypeptide, and detecting labeled hydroxyproline incorporation by analytical means, such as thin layer chromatography.

A further embodiment of the invention relates to methods of identifying or detecting prolyl hydroxylation wherein the substrate peptide (or polypeptide) is contacted with a source of prolyl hydroxylase in the presence or absence of putative inhibitors, and the degree of prolyl hydroxylation is monitored as described in any one of the above three paragraphs. In one embodiment, the peptide corresponds to HIF1 α 555-575, and the prolyl

hydroxylase consists of a mammalian cell extract. In another embodiment, the peptide corresponds to HIF1a 555-575 and the prolyl hydroxylase consists of purified or partially purified Egl9. Further, particularly useful embodiments relate to small molecule inhibitors of prolyl hydroxylation such as identified using this method, and use of the inhibitors to
5 treat diseases characterized by ischemia.

The percentage homology (also referred to as identity) of DNA and amino acid sequences can be calculated using commercially available algorithms. The following programs (provided by the National Center for Biotechnology Information) may be used to determine homologies: BLAST, gapped BLAST and PSI-BLAST, which may be used with
10 default parameters. The algorithm GAP (Genetics Computer Group, Madison, WI) uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of either of the terms "homology" and "homologous" herein does not imply any necessary evolutionary
15 relationship between compared sequences, in keeping for example with standard use of terms such as "homologous recombination" which merely requires that two nucleotide sequences be sufficiently similar to recombine under the appropriate conditions.

The precise format of the screening assays may be varied using routine skill and knowledge. The amount of VHL, HIF α subunit and, where required, further components,
20 may be varied depending upon the scale of the assay. In general, the person of skill in the art will select relatively equimolar amounts of the two components, say from 1:10 to 100:1, preferably from 1:1 to 10:1. molar ratio of VHL to HIF α subunit. However there may be particular assay formats which can be practiced outside this range.

Where assays of the invention are performed within cells, the cells may be treated to
25 provide or enhance a normoxic environment. By "normoxic" it is meant levels of oxygen similar to those found in normal air, e.g. about 21% O₂ and 5% CO₂, the balance being nitrogen. Of course, these exact proportions do not have to be used, and may be varied independently of each other. Generally a range of from 10-30% oxygen, 1-10% CO₂ and a balance of nitrogen or other relatively inert and non-toxic gas may be used. Normoxia may
30 be induced or enhanced in cells, for example by culturing the cells in the presence of hydrogen peroxide as described above.

Alternatively, or by way of controls, cells may also be cultured under hypoxic conditions. By "hypoxic" it is meant an environment with reduced levels of oxygen. Most

preferably oxygen levels in cell culture will be 0.1 to 1.0% for the provision of a hypoxic state. Hypoxia may be induced in cells simply by culturing the cells in the presence of lowered oxygen levels. The cells may also be treated with compounds which mimic hypoxia and cause up regulation of HIF α subunit expression. Such compounds include iron
5 chelators, cobalt (II), nickel (II) or manganese (II), all of which may be used at a concentration of 20 to 500 μ M. such as 100 μ M. Iron chelators include desferrioxamine, O-phenanthroline or hydroxypyridinones (e.g. 1,2-diethyl hydroxypyridinone (CP94) or 1,2-dimethyl hydroxypyridinone (CP20)).

Cells in which assays of the invention may be preformed include eukaryotic cells,
10 such as yeast, insect, mammalian, primate and human cells. Mammalian cells may be primary cells or transformed cells, including tumor cell lines. The cells may be modified to express or not to express other proteins which are known to interact with HIF (x subunit proteins and VHL protein, for example Elongin C and Elongin B proteins in the case of VHL and ARNT protein, in the case of HIF a subunit protein.)

15 In cell free systems such additional proteins may be included, for example by being provided by expression from suitable recombinant expression vectors.

In assays performed in cells, it will be desirable to achieve sufficient expression of VHL to recruit sufficient HIF α subunit to a complex such that the effect of a putative modulator compound may be measured. The level of expression of VHL and HIF α subunit
20 may be varied within fairly wide limits, so that the intracellular levels of the two may vary by a wide ratio, for example from 1:10 to 1000:1, preferably 1:1 to 100:1, molar ratio of VHL to HIF α subunit.

The amount of putative modulator compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of
25 compound used. Typically, from about 0.01 to 100 μ M concentrations of putative modulator compound may be used, for example from 0.1 to 10 μ M. Modulator compounds may be those which either agonize or antagonize the interaction. Antagonists (inhibitors) of the interaction are particularly desirable.

Modulator compounds which may be used may be natural or synthetic chemical
30 compounds used in drug screening programs. Extracts of plants which contain several characterized or uncharacterized components may also be used.

The invention relates in part to methods and compositions relating to detecting, localizing and quantifying enzyme activities and protein-protein interactions *in vivo*, *in vitro* and *in silico* using light-emitting fusion proteins. The fusion proteins contain domains capable of binding by enzymes and other ligands, and of being modified as a consequence of this binding. The light generating domains include, without limitation, regions from fluorescent proteins and bioluminescent proteins. Light emission is detected by known methods, such as detection with suitable instrumentation (such as a CCD camera) *in vivo*, *in vitro* or *in silico*, such as in a living cell or intact organism, a cell culture system, a tissue section, or an array.

Light-generating fusion proteins of the invention are capable of taking part in a luminescent reaction whereby different biological, biochemical, chemical and physical events are measured. The light-generating fusion protein is capable of being modified such that it does or does not emit light or cause light to be emitted. Light-generating fusion proteins include a ligand binding site and a light-generating polypeptide moiety, wherein the bioluminescence of the polypeptide moiety changes upon binding of a ligand at the ligand binding site.

Without wishing to be bound by interpretation, the ligand binding site acts as in a sense as a "switch" for the light-generating polypeptide moiety, i.e., when the ligand binds to the ligand binding site, the light-generating polypeptide moiety emits light, or alternately, ceases to do so upon ligand binding. The "switching" on or off, in embodiment, may be done by means of a "colinear effector site" which includes regions of the light-generating polypeptide moiety that, when acted on by events subsequent to ligand binding, cause the light-generating polypeptide moiety to change its present light-emitting state (i.e., on or off). The regions making up the colinear effector site may do this by, e.g., conformational distortion, chemical modification, e.g., ubiquitination of a residue or residues in the colinear effector site, or by cleavage of a portion of all or part of the colinear effector site.

The selection of a light-generating polypeptide moiety of the light-generating fusion protein should be done so as to produce light capable of penetrating animal tissue such that it can be detected externally in a non-invasive manner. The ability of light to pass through a medium such as animal tissue (composed mostly of water) is determined primarily by the light's intensity and wavelength.

The more intense the light produced in a unit volume, the easier the light will be to detect. The intensity of light produced in a unit volume depends on the spectral

characteristics of individual light-generating polypeptide moieties, and on the concentration of those moieties in the unit volume. Accordingly, schemes that place a high concentration of light-generating polypeptide moieties in or on an entity (such as high-efficiency loading of a liposome or high-level expression of a light-generating fusion protein in a cell)

- 5 typically produce brighter light-generating fusion proteins (LGPs), which are easier to detect through deeper layers of tissue, than schemes which conjugate, for example, only a single LGM onto each entity.

A second factor governing detectability through a layer of tissue is the wavelength of the emitted light. Water may be used to approximate the absorption characteristics of
10 animal tissue, since most tissues are composed primarily of water. It is well known that water transmits longer-wavelength light (in the red range) more readily than it does shorter wavelength light.

Accordingly, light-generating polypeptide moieties which emit light in the range of yellow to red (550-1100 nm) are typically preferable to those which emit at shorter
15 wavelengths. However, excellent results can be achieved in practicing the present invention with LGMs that emit in the range of 486 nm, despite the fact that this is not an optimal emission wavelength.

Fluorescence-based Moieties. Because fluorescent molecules require input of light in order to luminesce, their use in the invention may be more involved than the use of
20 bioluminescent molecules. Precautions are typically taken to shield the excitatory light so as not to contaminate the fluorescence photon signal being detected from the subject. Obvious precautions include the placement of an excitation filter at the radiation source. An appropriately-selected excitation filter blocks the majority of photons having a wavelength similar to that of the photons emitted by the fluorescent moiety. Similarly, a
25 barrier filter is employed at the detector to screen out most of the photons having wavelengths other than that of the fluorescence photons. Filters such as those described above can be obtained from a variety of commercial sources, including Omega Optical, Inc. (Brattleboro, Vt.).

Alternatively, a laser producing high intensity light near the appropriate excitation
30 wavelength, but not near the fluorescence emission wavelength, can be used to excite the fluorescent moieties. An x-y translation mechanism may be employed so that the laser can scan the subject, for example, as in a confocal microscope.

As an additional precaution, the radiation source may be placed behind the subject and shielded, such that the only radiation photons reaching the site of the detector are those that pass all the way through the subject. Furthermore, detectors may be selected that have a reduced sensitivity to wavelengths of light used to excite the fluorescent moiety.

5 An advantage of small fluorescent molecules is that they are less likely to interfere with the bioactivity of the entity to which they are attached than a would a larger light-generating moiety. In addition, commercially-available fluorescent molecules can be obtained with a variety of excitation and emission spectra that are suitable for use with the present invention. For example, Molecular Probes (Eugene, Oreg.) sells a number of
10 fluorophores, including Lucifer Yellow (abs. at 428 nm, and emits at 535 nm) and Nile Red (abs. at 551 nm and emits at 636 nm). Further, the molecules can be obtained derivatized with a variety of groups for use with various conjugation schemes (e.g., from Molecular Probes).

Bioluminescence-based Moieties. The subjects of chemiluminescence
15 (luminescence as a result of a chemical reaction) and bioluminescence (visible luminescence from living organisms) have, in many aspects, been thoroughly studied.

 An advantage of bioluminescent moieties over fluorescent moieties, is that there is virtually no background in the signal. The only light detected is light that is produced by the exogenous bioluminescent moiety. In contrast, the light used to excite a fluorescent
20 molecule often results in the fluorescence of substances other than the intended target. This is particularly true when the "background" is as complex as the internal environment of a living animal.

 Ligands include, in a preferred embodiment, enzymes which are capable of modifying a light-generating polypeptide moiety such that it does or ceases to emit light
25 when modified. In use, this happens when, e.g., the light-generating fusion protein comes in contact with a ligand on an entity or produced by the entity, and the ligand binds to the ligand binding site, altering the light-generating properties of the light-generating polypeptide moiety. Examples include the following.

 In an especially useful embodiment of the present invention, a light-generating
30 fusion protein comprising a binding site for an E3 ubiquitin ligase and a light-generating polypeptide moiety capable of being modified by E3 at a modification site can be used for diagnostic and treatment purposes. Ubiquitin ligases (e.g., E3) bind to colinear 'T' (see FIG. 1) present on their substrates. Examples include the SCF (Skp1/Cdc53/F-box)

ubiquitin ligases, the VBC (pVHL/elongin B/elongin C) ubiquitin ligase, and the MDM2 ubiquitin ligase. It is already established that light-generating proteins such as GFP and luciferase can be fused to heterologous polypeptides without loss of activity. In some embodiments, the light-generating polypeptide moiety may be modified to include a surface exposed lysine residue accessible as a ubiquitin acceptor site. In a First embodiment, a light-generating fusion protein of the invention may be used to monitor ischemia in living tissues and animals. The First embodiment comprises a polypeptide derived from the HIF1 α (hypoxia-inducible factor 1 α) which acts as a binding site for VBC. This binding site is only recognized by VBC in the presence of oxygen as a result of proline hydroxylation. The light-generating fusion protein of the First embodiment is advantageously stable in hypoxic cells, but unstable in well oxygenated cells. In a related embodiment, the HIF-derived polypeptide described above may be fused to a suicide moiety such as a protein (such as HSV TK or an adenoviral protein like E1A), which can be used in selective killing of ischemic cells (such as in a solid tumor).

Binding and/or modification sites are well-known in the art for a variety of kinases including cyclin-dependent kinases, ATM/ATR, JNK kinases, and receptor tyrosine kinases. In one embodiment, a light-generating fusion protein may be fused to a binding site for a kinase of interest. In some embodiments, it is useful to introduce one or more binding sites for a selected kinase into the light-generating fusion protein. By way of example, since phosphorylated serine, threonine, and tyrosine, by virtue of their negative charges, frequently mimic aspartic acid and/or glutamic acid residues, individual aspartic acid and glutamic acid residues are replaced with serine, threonine, or tyrosine (in the context of the kinase modification site). Such substitutions may be made singly and in combination. In another embodiment, the modification site can be empirically determined by carrying out a linker scan of the light-generating protein using a linker encoding the kinase modification site. In yet another embodiment the light-generating polypeptide moiety is mutagenized (either random or targeted mutagenesis) to generate modification sites in which the light-generating polypeptide moiety is selectively inactivated or activated by the kinase; this mutagenesis is performed using cells (such as yeast) rendered conditional for the kinase. In still yet another embodiment, a kinase modification site is designed *in silico* based on comparison of the binding site of the selected kinase to the primary sequence of the light-generating polypeptide moiety, coupled with knowledge of the three dimensional structure of the light-generating polypeptide moiety.

In an additional embodiment described below, a light-generating polypeptide moiety is fused to a polypeptide recognized by a cyclin/cdk2 complex. This enzyme phosphorylates serine or threonine with an absolute requirement for proline in the +1 position. By way of example, GFP contains 13 proline residues including one threonine-proline site and two aspartic acid-proline sites. In one embodiment, a light-generating protein (*e.g.*, GFP) is fused to a cyclin/cdk2 binding site such as Y-Lys-X₁-Leu-K-X₂-Leu-Y' (SEQ ID NO. 9). The light-generating protein is phosphorylated and inactivated by cyclin/cdk2, providing a detectable signal which is selectively off in the presence of cyclin/cdk2. In another embodiment, the light-generating polypeptide moiety is mutated so that it is not phosphorylated and activated by cyclin/cdk2. An example of this mutation would be to mutate the two aspartic acid-proline sites to serine (or threonine)-prolines.

In this additional embodiment, the ligand binding site is a polypeptide recognized by a cyclin/cdk2 complex, *e.g.*, comprising the amino acid sequence Y-Lys-X₁-Leu-K-X₂-Leu-Y', wherein X₁, X₂, are independently any one or more amino acids; and Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids.

An example of such a target peptide is RB: PKPLKKLRFD (SEQ ID NO: 1). In a related aspect of this additional embodiment, the ligand binding site comprises the amino acid sequence Y-X₁-Arg-Arg-Leu-Y', wherein X₁ is Lys or Cys; and Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids. Two non-limiting examples of the target peptide of this related aspect of this additional embodiment are: E2F1: GRPPVKRRDLLE (SEQ ID NO: 2); derived from the E2F1 protein, and p21: CCSKACRRLFGP (SEQ ID NO: 3), derived from the p21 protein.

The fusion protein of this additional embodiment can be produced by standard recombinant DNA techniques, discussed *supra*.

The ligand binding site of the light-generating fusion protein of this additional embodiment is derived from the retinoblastoma (RB) protein. Adams, et al., 1999 *Mol. Cell Biol.* 19:1068 describes RB protein. An RB polypeptide comprises 928 amino acid residues. As described in more detail below, this ligand binding site comprises a unique cyclin binding domain derived from RB. In the light-generating fusion protein described above, where present, Y comprises between 1 and 900 amino acid residues, preferably corresponding to the sequence of "N-terminal" RB amino acid residues 1-868. Thus, in a

preferred embodiment Y can represent any sequence of N-terminal amino acids of RB, for example residues 794 - 829, and so on up to and including residues 1-868. Y' can also comprise between 1 and 600 amino acid residues, preferably corresponding to the sequence of "C-terminal" RB amino acid residues 879-928. Thus, in a preferred embodiment, Y' can
5 represent any sequence of C-terminal amino acids of RB, for example 879-910, and so on up to and including residues 879-928. Y and Y' can also contain conservative substitutions of amino acids present in the N-terminal and C-terminal RB sequences described above. In a further preferred embodiment, Y and Y' are absent.

The light-generating fusion protein of this additional embodiment provides a way
10 for detecting "cancerous tissue" or tissue subject to aberrant cell proliferation and therefore at risk for cancer. In addition to tissue that becomes cancerous due to an *in situ* neoplasm, for example, the light-generating fusion protein also provides a method of detecting cancerous metastatic tissue present in distal organs and/or tissues. Thus such tissue may be detected by contacting tissue suspected of being cancerous with the light-generating fusion
15 protein under appropriate conditions to cause the phosphorylation of the light-generating fusion protein in cancerous tissue, thereby detecting the presence of cancerous tissue. The ligand binding site of the light-generating fusion protein of this embodiment provides a binding site for cyclin-cyclin-dependent kinase (cdk) complexes which phosphorylate the protein, causing it to emit or not emit light in the presence of active cycle-cdk complexes.
20 Under appropriate conditions, the light-generating fusion protein will therefore be phosphorylated or inactive in tissue that is not cancerous, and unphosphorylated and preserving its light-emitting properties in tissue that is cancerous, *e.g.* primary and metastatic tissue.

In another useful embodiment, a light-generating polypeptide moiety is fused to a
25 polypeptide recognized by a cyclin/cdk2 complex. This enzyme phosphorylates serine or threonine with an absolute requirement for proline in the +1 position. By way of example, GFP contains 13 proline residues including one threonine-proline site and two aspartic acid-proline sites. In one embodiment, a light-generating protein (*e.g.*, GFP) is fused to a cyclin/cdk2 binding site such as Y-Lys-X₁-Leu-K-X₂-Leu-Y' (SEQ ID NO. 9). The light-
30 generating protein is phosphorylated and inactivated by cyclin/cdk2, providing a detectable signal which is selectively off in the presence of cyclin/cdk2. In another embodiment, the light-generating polypeptide moiety is mutated so that it is not phosphorylated and activated by cyclin/cdk2. An example of this mutation would be to mutate the two aspartic acid-proline sites to serine (or threonine)-prolines.

Phosphorylation is one of the most important ways to posttranslationally modify proteins, and it regulates diverse cell physiological processes (transport, proliferation, differentiation). For example, phosphorylation is involved in all phases of cell division: in transition from G1 to S phase, progression of cells during S phase and entry into M phase.

- 5 The physiological function of oncoproteins and tumor suppressor proteins that are involved in gene expression and replication are also regulated by phosphorylation. Many growth factors and their receptors are encoded by oncogenes which are mutated or overexpressed in a variety of human tumors. Mutation or overexpression of these oncogenes leads to unchecked cell division, and transformation of normal cells to malignant. In an
- 10 embodiment of the present invention, a light-generating fusion protein may include a phosphatase binding site and a modification site, such as a phosphorylated amino acid residue capable of being modified by a protein "phosphatase".

- For most proteases, the enzyme binding site and modification site are largely congruent and can be encompassed in short peptides. In an embodiment, a light-generating
- 15 fusion protein may include a protease binding site and a modification site capable of being cleaved by the protease. The binding site and modification site may be the same site or in two discrete regions. In one embodiment, wherein the binding and modification site are congruent, linker scanning mutagenesis of a given light-generating protein is carried out using a linker that encodes the congruent binding/modification site. Resulting mutants are
- 20 those light-generating fusion proteins containing a binding/modification site that preserves light-emitting activity in protease deficient cells and exhibits protease sensitivity *in vitro* and *in vivo*. In the Third embodiment described herein, a light-generating fusion protein containing an HIV protease site is especially useful for monitoring the presence or absence of HIV (in this case, with HIV-positive cells not emitting light). In an alternative
- 25 embodiment, the light-generating fusion protein is fused via a linker to a protein that inhibits the light-emitting activity of the light-generating polypeptide moiety. The linker includes a binding/modification site for an HIV protease. Thus, cleavage at the binding/modification site should remove the inhibiting protein moiety, thus yielding a positive signal in cells that are HIV positive.

- 30 As shown generally in **FIG. 1A**, an embodiment of the fusion protein of the invention contains a ligand binding site "T", a reporter domain (e.g., light-generating polypeptide moiety) "R", and a modification site "X". Binding of enzyme "E" (the ligand) to target site "T" results in a modification of modification site "X", which causes the reporter domain "R" to either emit or not emit light.

The site "T" and modification site "X" may be separate and in *cis* on the fusion protein, as shown in **FIG. 1B**. Either "T" or "X" can be proximal or distal to the amino terminus of the fusion protein.

5 In another embodiment shown in **FIG. 1C**, wherein the "T" and the "X" of the fusion protein are congruent within a domain of the fusion protein. In related embodiments, the congruent "T/X" can be at the amino terminus, the carboxy terminus, or at neither terminus of the fusion protein.

In a further embodiment shown in **FIG. 1D**, the "T" is on a protein associated with the fusion protein containing the "X". In a related embodiment, target site "T" is on the fusion protein and modification site "X" is on a protein that is physically associated with the fusion protein, wherein modification of the associated protein results in the fusion protein either emitting or not emitting light. The depiction of site "T" and modification site "X" are not intended to be limiting. "T" can be at the amino terminus, the carboxy terminus, or at neither terminus of the fusion protein, and "X" can be at the amino terminus, 15 the carboxy terminus, or at neither terminus of the associated protein.

The invention also includes a fusion protein in which the ligand binding site sequence "T" is unknown. In this embodiment, e.g., as shown in **FIG. 2A**, the fusion protein includes a first hetero- or homo-dimerization domain ("HD1"). An enzyme "E" capable of modifying modification site "X" on the fusion protein is fused to a second 20 hetero- or homo-dimerization domain "HD2" that interacts with "HD1". In a related embodiment, the targeting site "T" for an enzyme "E" can be generated by inserting one or more polypeptide sequences derived from a random or non-random peptide library into the fusion protein.

In a related embodiment (**FIG. 2B**), a binding domain of a protein "A" containing 25 an enzyme target site "T" interacts with a binding domain "B" of a fusion protein, which results in enzyme "E" modifying the reporter domain "R" of fusion protein "B" at modification site "X" such that the fusion protein does or does not emit light or cause light to be emitted. In the First embodiment, the ligand binding site comprises a polypeptide derived from the HIF1 α (hypoxia-inducible factor 1 α) which acts as a binding site for VBC, e.g., the amino acid sequence Y-X₁-Leu-X₂-Pro_h-X₃-X₄-X₅-X₆-Y' (SEQ ID NO:11), 30 wherein

Pro_h is hydroxylated proline;

X₁, X₂, X₃, X₄, X₅, and X₆ are amino acids selected so as to not modify or alter VHL binding properties. X₁, X₂, X₄, X₅, and X₆ are desirably independently Gly, Ala, Val, Leu, Ile, Pro, Met, Phe, or Trp, and X₃ is desirably Ser, Thr, or Tyr; and

Y and Y' are independently present or absent and, if present, independently
5 comprise a peptide having from 1 to 600 amino acids.

In a preferred embodiment, the ligand binding site comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , wherein either or both of residues 402 and 564 are proline or hydroxylated proline. In a more preferred
10 embodiment, the ligand binding site comprises an 80 to 120, 20 to 30, 12 to 14, or 4 to 12 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 402 and/or 564, inclusive, of HIF1 α , wherein residues 402 and/or 564 is proline or hydroxylated proline. "Residues adjacent to and/or surrounding" is meant to include the relevant sequence of HIF1 α either before, after, or flanking, the specified residue, e.g., residue 564.

15 Such proteins may be used effectively as oxygen-sensing proteins. The fusion proteins may be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, e.g., by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-
20 in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently
25 be annealed and reamplified to generate a chimeric gene sequence (*see, e.g.,* Ausubel et al. (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, 1992).

The ligand binding site noted above is derived from HIF1 α protein. U.S. Patent No. 6,222,018, describes HIF protein and its preparation in substantially pure form. HIF is composed of subunits HIF1 α and an isoform HIF1 β . HIF1 α polypeptide comprises 826
30 amino acid residues. As described in more detail below, the ligand binding sites of this particular fusion protein comprises a unique ubiquitin ligase binding domain derived from HIF1 α . In this fusion protein, where present, Y comprises between 1 and 600 amino acid residue, preferably corresponding to the sequence of "N-terminal" HIF1 α amino acid

residues 1-555. Thus, in a preferred embodiment Y can represent any sequence of N-terminal amino acids of HIF1 α , for example residues 554-555, 553-555, 552-555, and so on up to and including residues 1-555. Y' can also comprises between 1 and 600 amino acid residues, preferably corresponding to the sequence of "C-terminal" HIF1 α amino acid residues 576-826. Thus, in a preferred embodiment, Y' can represent any sequence of C-terminal amino acids of HIF1 α , for example 576-577, 576-578, 576-579, and so on up to and including residues 576-826. Y and Y' can also contain conservative substitutions of amino acids present in the N-terminal and C-terminal HIF1 α sequences described above. In a preferred embodiment of the ligand binding site defined above, Y and Y' are absent. In a further preferred embodiment, X₁ is Met, X₂ is Leu, X₃ is Ala, X₄ is Tyr, X₅ is Pro, and X₆ is Met. In a particularly preferred embodiment of the fusion protein, the ligand binding site has the amino acid sequence Asp-Leu-Asp-Leu-Glu-Met-Leu-Ala-Pro_n-Tyr-Ile-Pro-Met-Asp-Asp-Asp-Phe-Gln-Leu-Arg, corresponding to HIF1 α amino acid residues 556-575, with a hydroxylated proline at amino acid residue 564.

The invention also provides a nucleic acid molecule encoding the fusion protein or polypeptide of the invention. (As used herein, the terms polypeptide and protein are interchangeable). An "isolated" nucleic acid molecule is one that is separated from other nucleic acid molecules that are present in the natural source of the nucleic acid. Examples of isolated nucleic acid molecules include, but are not limited to, recombinant DNA molecules contained in a vector, recombinant DNA molecules maintained in a heterologous host cell, partially or substantially purified nucleic acid molecules, and synthetic DNA or RNA molecules. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to the nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer. The present invention therefore also provides a vector comprising the nucleic acid of the invention. In a preferred embodiment, the vector further comprises a promoter operably linked to the

nucleic acid molecule. In a further preferred embodiment the invention provides a cell containing the vector of the invention.

The light-generating fusion protein of, e.g., the First embodiment provides a means for detecting hypoxic tissue or tissue subject to chronic hypoxia and therefore at risk for ischemia. In addition to tissue that becomes ischemic due to occurrence of a stroke, heart attack or embolism, for example, the fusion protein also provides a method of detecting ischemic tissue present in tumors. Thus the invention may be used to detect such tissue by contacting tissue suspected of being hypoxic with the light-generating fusion protein under appropriate conditions to cause the ubiquitination of the fusion protein in normoxic tissue, thereby detecting the presence of hypoxic tissue. As described more fully below, the ligand binding site of the light-generating fusion protein also provides a binding site for ubiquitin ligases which destroy the protein in the presence of oxygen. Under appropriate conditions, the light-generating fusion protein of the invention will therefore be "unstable" or destroyed in tissue that is not hypoxic, i.e., that is well oxygenated, and "stable" or preserving its light-emitting properties in tissue that is hypoxic, i.e. that lacks sufficient oxygen.

The Third embodiment provides a means for detecting "infected tissue" or tissue subject to contact with infectious agents and therefore at risk for infection, e.g., monitoring the presence or absence of HIV. The ligand binding site of the Third embodiment comprises a binding site for a protease, such as an infection-associated protease. For example, protease cleavage sites of the human immunodeficiency virus (HIV-1) protein precursor Pr55 (gag) protein include p2/NC, NC/p1, and NC/TFP.

The ligand binding site of the light-generating fusion protein of this Third embodiment may be derived from the HIV-1 protein. One non-limiting example of the target peptide of this Third embodiment is:

Y-GSGIF*LETSL-Y' (SEQ ID NO:6) (See Beck et al., (2000) Virology 274(2):391-401.

Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids, and "*" indicates the cleavage site of the fusion protein by a protease.

In addition to tissue that becomes infected due to an acute or chronic infection by an infectious agent, for example, the light-generating fusion protein may be used in detecting infected tissue present in distal organs and/or tissues, e.g., by contacting tissue suspected of being infected with the light-generating fusion protein of the invention under appropriate

conditions to cause the proteolysis of the light-generating fusion protein in infected tissue, thereby detecting the presence of infected tissue. The ligand binding site of the light-generating fusion protein of this Third embodiment provides a binding site for a protease which degrades or modifies the protein, causing it to emit or not emit light in the presence of one or more proteases. Under appropriate conditions, the light-generating fusion protein will therefore be proteolyzed or inactive in tissue that is infected (although there may be cases where proteolysis leads to light generation), and unproteolyzed and preserving its light-emitting properties in tissue that is infected.

A Fourth embodiment of a light-generating fusion protein of the invention comprises a light-generating protein moiety and a ligand binding site, wherein the ligand binding site comprises an amino acid sequence capable of binding to an "associated" protein. The association can occur by covalent or non-covalent binding. This associated protein may itself be a light-generating fusion protein, comprising a binding polypeptide capable of binding to the light-emitting light-generating fusion protein and an enzyme capable of modifying the light-emitting light-generating fusion protein.

A non-limiting example of the target peptide of this Fourth embodiment is:

HD1: WFHGKLSR (Amino acids 488-495 of Accession No. P29353, human SHC1; SEQ ID NO: 4).

This target polypeptide contains an SH2 domain. Therefore, any associated protein with an SH2 domain should interact with the target peptide of the Fourth embodiment. A non-limiting example of the binding peptide of the associated protein of this Fourth embodiment is:

HD2: WNVGSSNR (Amino acids 624-631 of Accession No. P27986; human PI3K p85 subunit; SEQ ID NO: 5).

Another non-limiting example of the Fourth embodiment would be to fuse the FK506 binding protein (FKBP12) domain moiety to GFP and the FRAP domain moiety to Skpl or elonginC. Therefore in the presence of rapamycin, which promotes the high affinity interaction of FKBP12 and FRAP, the core E3 ligase machinery would bind to and destroy GFP, eliminating the bioluminescence wherever rapamycin is present.

The ligand binding site of the light-generating fusion protein of this Fourth embodiment is derived from the human SHC1 proteinprotein. See Pelicci et al., (1992) *Cell*

70:93-104, describes the SHC1 protein with an SH2 domain which is implicated in mitogenic signal transduction.

The light-generating fusion protein of this Fourth embodiment provides a means for detecting enzymatic activity where the enzyme binding site is undefined.

5 Cyclin/cdk activity may be assayed in another embodiment of the invention wherein a test sample is contacted with a light-generating fusion protein comprising a cyclin/cdk binding site and a light-generating polypeptide moiety; and thereafter the presence or amount of cyclin/cdk activity in said test sample is determined by measuring the luminescence of the test sample.

10 The invention yet further relates to DNA constructs, including an isolated DNA encoding a modified LGP wherein one or more amino acids have been substituted, inserted or deleted to provide a ligand binding site such as a ubiquitin ligase or protease recognition site, wherein fluorescence of the LGP changes upon binding of a ligand to the ligand binding site.

15 The invention also relates to light-generating fusion proteins having a ligand binding site, such as a ubiquitin ligase binding site, or a HIF1 α polypeptide moiety; and a light-generating polypeptide moiety. Another embodiment features a light-generating fusion protein with a cyclin/cdk binding site, a suicide protein polypeptide moiety, and a light-generating polypeptide moiety; and a light-generating fusion protein comprising a protein
20 dimerization domain and a light-generating protein moiety. In a preferred embodiment, the light-generating fusion proteins have the property that upon ligand binding to the ligand binding site, the luminescence of the light-generating polypeptide moiety is changed without altering the phosphorylational state of the fusion protein.

The invention further relates to fusion proteins including a HIF1 α polypeptide
25 moiety or cyclin/cdk binding site, and a suicide protein polypeptide moiety. A light-generating polypeptide moiety may optionally be included. These protein constructs may be used to selectively target certain cells, e.g., hypoxic tumor cells, for destruction. For example, the invention includes methods of treating hypoxic or ischemic disorders by administering to a subject an effective amount of a fusion protein comprising a HIF α
30 polypeptide moiety having a binding affinity for prolyl hydroxylase, and a suicide polypeptide moiety, such that the hypoxic or ischemic disorder is treated. Methods of killing hypoxic tumor cells, wherein an effective amount of the fusion/suicide protein is

administered to a subject, such that the hypoxic tumor cells are killed; and methods of treating cell-proliferating disorders by administering to a subject an effective amount of a fusion protein comprising a cyclin/cdk binding site and a suicide protein polypeptide moiety, such that the cell-proliferating disorder is treated, are also contemplated.

5 Other screening methods are also part of the invention. For example, modulators of activity or latency of, or predisposition to disorders may be identified by administering a test compound to a test animal at increased risk for a disorder (e.g., cancer, diabetes, heart disease, stroke, or a hypoxia-related disorder), wherein the test animal recombinantly expresses a light-generating fusion protein comprising a ligand binding site and a light-
10 generating polypeptide moiety, wherein the light generation of the light-generating fusion protein changes upon binding of a ligand at the ligand binding site, and the ligand binding site recognizes a ligand on an entity associated with a disorder, or a product of the disorder; allowing for localization of the light-generating fusion protein and an entity, wherein contact between the ligand binding site and a ligand associated with the disorder causes a
15 modification of a colinear effector site which alters the light generation of the light-generating polypeptide moiety; detecting the luminescence of the light-generating polypeptide moiety in the test animal after administering the compound; and comparing the luminescence of the light-generating polypeptide moiety in the test animal with the luminescence of the light-generating polypeptide moiety in a control animal, wherein a
20 change in the activity of the light-generating polypeptide moiety in the test animal relative to the control indicates the test compound is a modulator of latency of or predisposition to, the disorder in question.

The invention advantageously may be used to non-invasively determine the effects of an anti-hypoxic compound *in vivo*. A light-generating fusion protein of the invention, or
25 a cell expressing same, comprising an ubiquitin ligase binding site and a light-generating polypeptide moiety, wherein the light generation of the light-generating fusion protein changes upon binding of a ubiquitin ligase at the ubiquitin ligase binding site, the ubiquitin ligase binding site recognizing a ubiquitin ligase present in hypoxic conditions in hypoxic tissue is administered to a subject. Localization of the light-generating fusion protein or
30 cell in hypoxic tissue in the subject (wherein contact between the ubiquitin ligase binding site and a ubiquitin ligase causes a modification of a colinear effector site which alters the light generation of the light-generating polypeptide moiety) is allowed to occur, and the ability of the candidate compound to inhibit hypoxia is determined, by measuring the luminescence of the localized light-generating fusion protein.

The test compounds of the invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds. See, e.g., Lam, 1997. *Anticancer Drug Design* 12: 145.

Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be screened with any of the assays of the invention. Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt, *et al.*, 1993. *Proc. Natl. Acad. Sci. U.S.A.* 90: 6909; Erb, *et al.*, 1994. *Proc. Natl. Acad. Sci. U.S.A.* 91: 11422; Zuckermann, *et al.*, 1994. *J. Med. Chem.* 37: 2678; Cho, *et al.*, 1993. *Science* 261: 1303; Carrell, *et al.*, 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2059; Carrell, *et al.*, 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2061; and Gallop, *et al.*, 1994. *J. Med. Chem.* 37: 1233.

Libraries of compounds may be presented in solution (e.g., Houghten, 1992. *Biotechniques* 13: 412-421), or on beads (Lam, 1991. *Nature* 354: 82-84), on chips (Fodor, 1993. *Nature* 364: 555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner, U.S. Patent 5,233,409), plasmids (Cull, *et al.*, 1992. *Proc. Natl. Acad. Sci. USA* 89: 1865-1869) or on phage (Scott and Smith, 1990. *Science* 249: 386-390; Devlin, 1990. *Science* 249: 404-406; Cwirla, *et al.*, 1990. *Proc. Natl. Acad. Sci. U.S.A.* 87: 6378-6382; Felici, 1991. *J. Mol. Biol.* 222: 301-310; Ladner, U.S. Patent No. 5,233,409.).

The invention provides methods for determining hypoxic conditions, cancer or infection in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.) Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs, compounds) on hypoxic conditions, cancer or infection in clinical trials.

Thus, the diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with

hypoxic conditions, cancer or infection. Furthermore, the prognostic assays described herein can be used to determine whether a subject should be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with hypoxic conditions, cancer or
5 infection.

The pharmaceutical compositions of the invention comprise the novel agents combined with a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like,
10 compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils
15 may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with
20 its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (*i.e.*, topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols,
25 glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.
30 The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous

preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable

5 under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a

10 coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol,

15 sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients

20 enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active

25 ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral

therapeutic administration, the active compound can be incorporated with excipients and

30 used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The

tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be

treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular
5 therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The invention includes entities which may have been modified or conjugated to include a light-generating fusion protein of the invention. Such conjugated or modified entities are referred to as light-emitting entities, or simply conjugates. The conjugates
10 themselves may take the form of, for example, molecules, macromolecules, particles, microorganisms, or cells. The methods used to conjugate a light-generating fusion protein to an entity depend on the nature of the light-generating fusion protein and the entity. Exemplary conjugation methods are discussed in the context of the entities described below.

15 **Small molecules.** Small molecule entities which may be useful in the present invention include compounds which specifically interact with a pathogen or an endogenous ligand or receptor. Examples of such molecules include, but are not limited to, drugs or therapeutic compounds; toxins, such as those present in the venoms of poisonous organisms, including certain species of spiders, snakes, scorpions, dinoflagellates, marine
20 snails and bacteria; growth factors, such as NGF, PDGF, TGF and TNF; cytokines; and bioactive peptides.

The small molecules are preferably conjugated to light-generating fusion proteins in a way that that the bioactivity of the small molecule is not substantially affected. Conjugations are typically chemical in nature, and can be performed by any of a variety of
25 methods known to those skilled in the art.

Small molecules conjugated to light-generating fusion proteins of the present invention may be used either in animal models of human conditions or diseases, or directly in human subjects to be treated. For example, a small molecule which binds with high affinity to receptor expressed on tumor cells may be used in an animal model to localize and
30 obtain size estimates of tumors, and to monitor changes in tumor growth or metastasis following treatment with a putative therapeutic agent. Such molecules may also be used to monitor tumor characteristics, as described above, in cancer patients.

Macromolecules. Macromolecules, such as polymers and biopolymers, constitute another example of entities useful in practicing the present invention. Exemplary macromolecules include antibodies, antibody fragments, light-generating fusion proteins and certain vector constructs.

5 Antibodies or antibody fragments, purchased from commercial sources or made by methods known in the art, can be used to localize their antigen in a mammalian subject by conjugating the antibodies to a light-generating polypeptide moiety, administering the conjugate to a subject by, for example, injection, allowing the conjugate to localize to the site of the antigen, and imaging the conjugate.

10 Antibodies and antibody fragments have several advantages for use as entities in the present invention. By their nature, they constitute their own targeting moieties. Further, their size makes them amenable to conjugation with several types of light-generating fusion proteins, including small fluorescent molecules and fluorescent and bioluminescent proteins, yet allows them to diffuse rapidly relative to, for example, cells or liposomes.

15 The light-generating fusion proteins can be conjugated directly to the antibodies or fragments, or indirectly by using, for example, a fluorescent secondary antibody. Direct conjugation can be accomplished by standard chemical coupling of, for example, a fluorophore to the antibody or antibody fragment, or through genetic engineering. Chimeras, or fusion proteins can be constructed which contain an antibody or antibody
20 fragment coupled to a fluorescent or bioluminescent protein. For example, Casadei, et al., describe a method of making a vector construct capable of expressing a fusion protein of aequorin and an antibody gene in mammalian cells.

 Conjugates containing antibodies can be used in a number of applications of the present invention. For example, a labeled antibody directed against E-selection, which is
25 expressed at sites of inflammation, can be used to localize the inflammation and to monitor the effects of putative anti-inflammatory agents.

 Vector constructs by themselves can also constitute macromolecular entities applicable to the present invention. For example, a eukaryotic expression vector can be constructed which contains a therapeutic gene and a gene encoding a light-generating
30 molecule under the control of a selected promoter (i.e., a promoter which is expressed in the cells targeted by the therapeutic gene). Expression of the light-generating molecule, assayed using methods of the present invention, can be used to determine the location and level of expression of the therapeutic gene. This approach may be particularly useful in

cases where the expression of the therapeutic gene has no immediate phenotype in the treated individual or animal model.

Viruses. Another entity useful for certain aspects of the invention are viruses. As many viruses are pathogens which infect mammalian hosts, the viruses may be conjugated to a light-generating fusion protein and used to study the initial site and spread of infection. In addition, viruses labeled with a light-generating fusion protein may be used to screen for drugs which inhibit the infection or the spread of infection.

A virus may be labeled indirectly, either with an antibody conjugated to a light-generating fusion protein, or by, for example, biotinylating virions as known in the art and then exposing them to streptavidin linked to a detectable moiety, such as a fluorescent molecule.

Alternatively, virions may be labeled directly with a fluorophore like rhodamine, using methods known in the art. The virus can also be genetically engineered to express a light-generating fusion protein. Labeled virus can be used in animal models to localize and monitor the progression of infection, as well as to screen for drugs effective to inhibit the spread of infection. For example, while herpes virus infections are manifested as skin lesions, this virus can also cause herpes encephalitis. Such an infection can be localized and monitored using a virus labeled by any of the methods described above, and various antiviral agents can be tested for efficacy in central nervous system (CNS) infections.

Particles. Particles, including beads, liposomes and the like, constitute another entity useful in the practice of the present invention. Due to their larger size, particles may be conjugated with a larger number of light-generating fusion proteins than, for example, can small molecules. This results in a higher concentration of light emission, which can be detected using shorter exposures or through thicker layers of tissue. In addition, liposomes can be constructed to contain an essentially pure targeting moiety, or ligand, such as an antigen or an antibody, on their surface. Further, the liposomes may be loaded with, for example, light-generating protein molecules, to relatively high concentrations.

Furthermore, two types of liposomes may be targeted to the same cell type such that light is generated only when both are present. For example, one liposome may carry luciferase, while the other carries luciferin. The liposomes may carry targeting moieties, and the targeting moieties on the two liposomes may be the same or different. Viral proteins on infected cells can be used to identify infected tissues or organs. Cells of the immune system can be localized using a single or multiple cell surface markers.

The liposomes are preferably surface-coated, e.g., by incorporation of phospholipid-polyethyleneglycol conjugates, to extend blood circulation time and allow for greater targeting via the bloodstream. Liposomes of this type are well known.

5 **Cells.** Cells, both prokaryotic and eukaryotic, constitute another entity useful in the practice of the present invention. Like particles, cells can be loaded with relatively high concentrations of light-generating moieties, but have the advantage that the light-generating moieties can be provided by, for example, a heterologous genetic construct used to transfect the cells. In addition, cells can be selected that express "targeting moieties", or molecules effective to target them to desired locations within the subject. Alternatively, the cells can
10 be transfected with a vector construct expressing an appropriate targeting moiety.

 The cell type used depends on the application. For example, bacterial cells can be used to study the infective process, and to evaluate the effects of drugs or therapeutic agents on the infective process with a high level of temporal and spatial resolution. Bacterial cells constitute effective entities. For example, they can be easily transfected to express a high
15 levels of a light-generating fusion protein, as well as high levels of a targeting protein. In addition, it is possible to obtain *E. coli* libraries containing bacteria expressing surface-bound antibodies which can be screened to identify a colony expressing an antibody against a selected antigen (Stratagene, La Jolla, Calif.). Bacteria from this colony can then be transformed with a second plasmid containing a gene for a light-generating protein, and
20 transformants can be utilized in the methods of the present invention, as described above, to localize the antigen in a mammalian host.

 Pathogenic bacteria can be conjugated to a light-generating fusion protein and used in an animal model to follow the infection process *in vivo* and to evaluate potential anti-infective drugs, such as new antibiotics, for their efficacy in inhibiting the infection.

25 Eukaryotic cells are also useful as entities in aspects of the present invention. Appropriate expression vectors, containing desired regulatory elements, are commercially available. The vectors can be used to generate constructs capable of expressing desired light-generating proteins in a variety of eukaryotic cells, including primary culture cells, somatic cells, lymphatic cells, etc. The cells can be used in transient expression studies, or,
30 in the case of cell lines, can be selected for stable transformants.

 Expression of the light-generating protein in transformed cells can be regulated using any of a variety of selected promoters. For example, if the cells are to be used as light-emitting entities targeted to a site in the subject by an expressed ligand or receptor, a

constitutively-active promoter, such as the CMV or SV40 promoter may be used. Cells transformed with such a construct can also be used to assay for compounds that inhibit light generation, for example, by killing the cells.

Alternatively, the transformed cells may be administered such they become
5 uniformly distributed in the subject, and express the light-generating fusion protein only under certain conditions, such as upon infection by a virus or stimulation by a cytokine. Promoters that respond to factors associated with these and other stimuli are known in the art. In a related aspect, inducible promoters, such as the Tet system can be used to transiently activate expression of the light-generating protein.

10 For example, CD4+ lymphatic cells can be transformed with a construct containing tat-responsive HIV LTR elements, and used as an assay for infection by HIV. Cells transformed with such a construct can be introduced into SCID-hu mice and used as model for human HIV infection and AIDS.

Tumor cell lines transformed to express the light-generating fusion protein, for
15 example, with a constitutively-active promoter, may be used to monitor the growth and metastasis of tumors. Transformed tumor cells may be injected into an animal model, allowed to form a tumor mass, and the size and metastasis of the tumor mass monitored during treatment with putative growth or metastasis inhibitors. Tumor cells may also be generated from cells transformed with constructs containing regulatable promoters, whose
20 activity is sensitive to various infective agents, or to therapeutic compounds.

Cell Transformation. Transformation methods for both prokaryotic cells and eukaryotic cells are well known in the art. Vectors containing the appropriate regulatory elements and multiple cloning sites are widely commercially available (e.g., Stratagene, La Jolla, Calif., or Clontech, Palo Alto, Calif.).

25 In another aspect, the present invention includes transgenic animals containing a heterologous gene construct encoding a light-generating fusion protein or complex of proteins. The construct is driven by a selected promoter, and can include, for example, various accessory proteins required for the functional expression of the light-generating protein, as well as selection markers and enhancer elements.

30 Activation of the promoter results in increased expression of the genes encoding the light-generating fusion proteins and accessory proteins. Activation of the promoter is achieved by the interaction of a selected biocompatible entity, or parts of the entity, with the

promoter elements. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein.

Light-generating fusion proteins are typically administered to a subject by any of a variety of methods, allowed to localize within the subject, and imaged. Since the imaging, or measuring photon emission from the subject, may last up to tens of minutes, the subject is desirably immobilized during the imaging process. Imaging of the light-generating polypeptide moiety involves the use of, e.g., a photodetector capable of detecting extremely low levels of light--typically single photon events--and integrating photon emission until an image can be constructed. Examples of such sensitive photodetectors include devices that intensify the single photon events before the events are detected by a camera, and cameras (cooled, for example, with liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of the subject to provide a frame of reference for the source of the emitted photons (i.e., localize the light-generating fusion proteins with respect to the subject). Such a "composite" image is then analyzed to determine the location and/or amount of a target in the subject.

Light-generating fusion proteins that have localized to their intended sites in a subject may be imaged in a number of ways. Guidelines for such imaging, as well as specific examples, are described below.

Localization of Light-generating fusion proteins. In the case of "targeted" conjugates, that is, conjugates which contain a targeting moiety--a molecule or feature designed to localize the conjugate within a subject or animal at a particular site or sites, localization refers to a state when an equilibrium between bound, "localized", and unbound, "free" entities within a subject has been essentially achieved. The rate at which such an equilibrium is achieved depends upon the route of administration. For example, a conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection. On the other hand, a conjugate administered orally to localize an infection in the intestine may take hours to achieve localization.

Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered. In a related aspect, localization of, for example, injected tumors cells expressing a light-generating

moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

By way of another example, localization is achieved when an entity becomes distributed following administration. For example, in the case of a conjugate administered to measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art. Furthermore, the state of localization as a function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

The "photodetector device" used should have a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, and to use the signal from such a device to construct an image.

In cases where it is possible to use light-generating moieties which are extremely bright, and/or to detect light-generating fusion proteins localized near the surface of the subject or animal being imaged, a pair of "night-vision" goggles or a standard high-sensitivity video camera, such as a Silicon Intensified Tube (SIT) camera (e.g. Hamamatsu Photonic Systems, Bridgewater, N.J.), may be used. More typically, however, a more sensitive method of light detection is required.

In extremely low light levels the photon flux per unit area becomes so low that the scene being imaged no longer appears continuous. Instead, it is represented by individual photons which are both temporally and spatially distinct from one another. Viewed on a monitor, such an image appears as scintillating points of light, each representing a single detected photon. By accumulating these detected photons in a digital image processor over time, an image can be acquired and constructed. In contrast to conventional cameras where the signal at each image point is assigned an intensity value, in photon counting imaging the amplitude of the signal carries no significance. The objective is to simply detect the presence of a signal (photon) and to count the occurrence of the signal with respect to its position over time.

At least two types of photodetector devices, described below, can detect individual photons and generate a signal which can be analyzed by an image processor. Reduced-

Noise Photodetection Devices achieve sensitivity by reducing the background noise in the photon detector, as opposed to amplifying the photon signal. Noise is reduced primarily by cooling the detector array. The devices include charge coupled device (CCD) cameras referred to as "backthinned", cooled CCD cameras. In the more sensitive instruments, the cooling is achieved using, for example, liquid nitrogen, which brings the temperature of the CCD array to approximately -120°C . "Backthinned" refers to an ultra-thin backplate that reduces the path length that a photon follows to be detected, thereby increasing the quantum efficiency. A particularly sensitive backthinned cryogenic CCD camera is the "TECH 512", a series 200 camera available from Photometrics, Ltd. (Tucson, Ariz.).

"Photon amplification devices" amplify photons before they hit the detection screen. This class includes CCD cameras with intensifiers, such as microchannel intensifiers. A microchannel intensifier typically contains a metal array of channels perpendicular to and co-extensive with the detection screen of the camera. The microchannel array is placed between the sample, subject, or animal to be imaged, and the camera. Most of the photons entering the channels of the array contact a side of a channel before exiting. A voltage applied across the array results in the release of many electrons from each photon collision. The electrons from such a collision exit their channel of origin in a "shotgun" pattern, and are detected by the camera.

Even greater sensitivity can be achieved by placing intensifying microchannel arrays in series, so that electrons generated in the first stage in turn result in an amplified signal of electrons at the second stage. Increases in sensitivity, however, are achieved at the expense of spatial resolution, which decreases with each additional stage of amplification. An exemplary microchannel intensifier-based single-photon detection device is the C2400 series, available from Hamamatsu.

Image Processors process signals generated by photodetector devices which count photons in order to construct an image which can be, for example, displayed on a monitor or printed on a video printer. Such image processors are typically sold as part of systems which include the sensitive photon-counting cameras described above, and accordingly, are available from the same sources. The image processors are usually connected to a personal computer, such as an IBM-compatible PC or an Apple Macintosh (Apple Computer, Cupertino, Calif.), which may or may not be included as part of a purchased imaging system. Once the images are in the form of digital files, they can be manipulated by a

variety of image processing programs (such as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View, Calif.) and printed.

The Detection Field Of The Device is defined as the area from which consistent measurements of photon emission can be obtained. In the case of a camera using an optical
5 lens, the detection field is simply the field of view accorded to the camera by the lens. Similarly, if the photodetector device is a pair of "night vision" goggles, the detection field is the field of view of the goggles.

Alternatively, the detection field may be a surface defined by the ends of fiber-optic cables arranged in a tightly-packed array. The array is constructed to maximize the area
10 covered by the ends of the cables, as opposed to void space between cables, and placed in close proximity to the subject. For instance, a clear material such as plexiglass can be placed adjacent the subject, and the array fastened adjacent the clear material, opposite from the subject.

The fiber-optic cable ends opposite the array can be connected directly to the
15 detection or intensifying device, such as the input end of a microchannel intensifier, eliminating the need for a lens. An advantage of this method is that scattering and/or loss of photons is reduced by eliminating a large part of the air space between the subject and the detector, and/or by eliminating the lens. Even a high-transmission lens transmits only a fraction of the light reaching the front lens element.

20 With higher-intensity LGPs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence. Other approaches may be used to generate three-
25 dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a light-emitting conjugate known to be localized in a particular region of the subject, only light
30 from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

Immobilizing the subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly agitated, no special immobilization precautions may be required, except to insure that the subject is in the field of the detection device at the start of the measuring period.

If, on the other hand, the photon emission measurement takes longer than about 20 msec, and the subject is agitated, precautions to insure immobilization of the subject during photon emission measurement, commensurate with the degree of agitation of the subject, need to be considered to preserve the spatial information in the constructed image. For example, in a case where the subject is a person and photon emission measurement time is on the order of a few seconds, the subject may simply be asked to remain as still as possible during photon emission measurement (imaging). On the other hand, if the subject is an animal, such as a mouse, the subject can be immobilized using, for example, an anesthetic or a mechanical restraining device.

In cases where it is desired to measure only the total amount of light emanating from a subject or animal, the subject does not necessarily need to be immobilized, even for long periods of photon emission measurements. All that is required is that the subject be confined to the detection field of the photodetector during imaging. It will be appreciated, however, that immobilizing the subject during such measuring may improve the consistency of results obtained, because the thickness of tissue through which detected photons pass will be more uniform from animal to animal.

Further Considerations During Imaging

The visualization of fluorescent light-generating moieties requires an excitation light source, as well as a photodetector. Furthermore, it will be understood that the excitation light source is turned on during the measuring of photon emission from the light-generating moiety.

Appropriate selection of a fluorophore, placement of the light source and selection and placement of filters, all of which facilitate the construction of an informative image, are discussed above, in the section on fluorescent light-generating moieties.

High-Resolution Imaging. Photon scattering by tissue limits the resolution that can be obtained by imaging LGMs through a measurement of total photon emission. It will be understood that the present invention also includes embodiments in which the light-generation of LGMs is synchronized to an external source which can be focused at selected points within the subject, but which does not scatter significantly in tissue, allowing the construction of higher-resolution images. For example, a focused ultrasound signal can be used to scan, in three dimensions, the subject being imaged. Light-generation from areas which are in the focal point of the ultrasound can be resolved from other photon emission by a characteristic oscillation imparted to the light by the ultrasound.

Constructing an Image of Photon Emission. In cases where, due to an exceptionally bright light-generating moiety and/or localization of light-generating fusion proteins near the surface of the subject, a pair of "night-vision" goggles or a high sensitivity video camera was used to obtain an image, the image is simply viewed or displayed on a video monitor. If desired, the signal from a video camera can be diverted through an image processor, which can store individual video frames in memory for analysis or printing, and/or can digitize the images for analysis and printing on a computer.

Alternatively, if a photon counting approach is used, the measurement of photon emission generates an array of numbers, representing the number of photons detected at each pixel location, in the image processor. These numbers are used to generate an image, typically by normalizing the photon counts (either to a fixed, pre-selected value, or to the maximum number detected in any pixel) and converting the normalized number to a brightness (greyscale) or to a color (pseudocolor) that is displayed on a monitor. In a pseudocolor representation, typical color assignments are as follows. Pixels with zero photon counts are assigned black, low counts blue, and increasing counts colors of increasing wavelength, on up to red for the highest photon count values. The location of colors on the monitor represents the distribution of photon emission, and, accordingly, the location of light-generating fusion proteins.

In order to provide a frame of reference for the conjugates, a greyscale image of the (still immobilized) subject from which photon emission was measured is typically constructed. Such an image may be constructed, for example, by opening a door to the imaging chamber, or box, in dim room light, and measuring reflected photons (typically for a fraction of the time it takes to measure photon emission). The greyscale image may be constructed either before measuring photon emission, or after. The image of photon

emission is typically superimposed on the greyscale image to produce a composite image of photon emission in relation to the subject.

If it is desired to follow the localization and/or the signal from a light-emitting conjugate over time, for example, to record the effects of a treatment on the distribution and/or localization of a selected biocompatible moiety, the measurement of photon emission, or imaging can be repeated at selected time intervals to construct a series of images. The intervals can be as short as minutes, or as long as days or weeks.

Analysis of Photon Emission Images Images generated by methods and/or using compositions of the present invention may be analyzed by a variety of methods. They range from a simple visual examination, mental evaluation and/or printing of a hardcopy, to sophisticated digital image analysis. Interpretation of the information obtained from an analysis depends on the phenomenon under observation and the entity being used.

Applications: Localization of Tumor Cells

The growth and metastatic spread of tumors in a subject may be monitored using methods and compositions of the present invention. In particular, in cases where an individual is diagnosed with a primary tumor, LGPs directed against the cells of the tumor can be used to both define the boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites. For example, LGPs, such as liposomes containing antibodies directed against tumor antigens and loaded with LGPs, can be administered to a subject, allowed to bind to tumor cells in the subject, imaged, and the areas of photon emission can be correlated with areas of tumor cells.

In a related aspect, images utilizing tumor-localizing LGPs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in inhibiting tumor growth or metastasis.

In the practice of the invention, the tissue and the light-generating fusion protein can be contacted *in vitro*, such as where one or more biological samples (*e.g.*, blood, serum, cells, tissue) are arrayed on a substrate under tissue culture conditions known by those in the art to preserve the viability of the tissue and then the fusion protein is added to the tissue culture. In a preferred embodiment of the methods of the invention the tissue is mammalian tissue, in particular human tissue.

The methods of the invention can also be practiced *in vivo* wherein the biological sample and the light-generating fusion protein are contacted by administration of the fusion protein (or a vector encoding the same) to a subject suspected of containing ischemic tissue under conditions to allow detection of the fusion protein in ischemic tissue present in the
5 subject. The invention thus also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. . . . Accordingly, one aspect of the invention relates to diagnostic assays for determining the presence of ischemic tissue in a biological sample to thereby determine whether an
10 individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with hypoxic conditions. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing conditions arising from ischemia.

Referring now to the Drawings, **FIG 1A** is a schematic representation of different
15 fusion proteins of the present invention. "T" indicates the enzyme target site on the fusion protein, "E" indicates the enzyme, "X" indicates the modification site on the fusion protein, and "R" indicates the reporter domain of the fusion protein. The modification at "X" by "E" results in the fusion protein becoming active or inactive "On/Off". **FIG 1B** shows an embodiment wherein the "T" and the "X" are separate domains and are in *cis* on the fusion
20 protein. **FIG 1C** shows an embodiment of the invention wherein the "T" and the "X" of the fusion protein are congruent within a domain of the fusion protein. **FIG 1D** shows an embodiment of the invention wherein the "T" is on a protein associated with the fusion protein containing the "X".

FIG 2 is a schematic representation of different fusion proteins of the present
25 invention. **FIG. 2A** is a schematic representation of a fusion protein and associated protein of the present invention in which a fusion protein and an associated protein "A" contains a known homo- or hetero-dimerization domain "HD" corresponding to the enzyme target site of an enzyme, where binding of the enzyme-associated "A" to the HD on the fusion protein results in modification of the fusion protein at "X". **FIG 2B** is a related aspect of the
30 invention in which a binding domain of a protein "A" containing an enzyme target site "T" interacts with a binding domain "B" of a fusion protein, which results in enzyme "E" modifying the reporter domain "R" of fusion protein "B" at modification site "X" such that the fusion protein does or does not emit light or cause light to be emitted.

FIG 3 shows pVHL binding to a modified form of HIF. (A) shows pVHL-defective renal carcinoma cells treated with increasing amounts of desferoxamine (2, 10, 100, 1000 μ m) or cobalt chloroxide (2, 10, 100, 1000 μ m) and immunoprecipitated with control (lane 1) or anti-HIF2 α antibody. Bound proteins were detected by anti-HIF2 α immunoblot (IB) or by farwestern (FW) analysis with purified pVHL/elongin B/elongin C (VBC) complexes. (B) shows VBC farwestern and anti-HIF2 α immunoblot analysis of ts20 cells grown at the restrictive temperature under hypoxic or normoxic conditions. (C and D) depict GST-HIF1 α (530-652), containing the oxygen-dependent degradation domain (ODD), produced in *E. Coli*, recovered on glutathione Sepharose, and incubated with rabbit reticulocyte lysate for 90 min at 30°C. In (D, lane 3), the reticulocyte lysate was first heat inactivated for 20 min. Following stringent washes the GST-ODD protein was subjected to VBC farwestern and anti-GST immunoblot analysis.

FIG 4 shows pVHL binding to a HIF1 α -derived peptide if Leu562 and Pro564 are intact. (A) shows binding of the indicated ³⁵S-labeled Gal4-HIF1 α fusion proteins to immobilized GST-pVHL, elongin B, elongin C complexes. (B) shows binding of ³⁵S-labeled pVHL to biotinylated HIF1 α (556-575) peptides with the indicated substitutions of residues 561-568. '+' indicates preincubation of peptide with unprogrammed reticulocyte lysate prior to addition of pVHL. (C and D) depict ³⁵S-labeled wild-type (WT), Pro564Ala, and Leu562Ala full-length HA-HIF1 α (panel C) and Gal4-HA-HIF1 α (530-652)(panel D) proteins immunoprecipitated with anti-HA antibody or captured with immobilized GST-VBC complexes. WG= wheat germ extract ; Retic = rabbit reticulocyte lysate.

FIG 5 shows ubiquitination and degradation of HIF linked to Leu562 and Pro 564. (A) depicts *in vitro* ubiquitination of ³⁵S-labeled wild-type, Leu562A, and Pro564A Gal4-HA-HIF1 α (530-652) in the presence of S100 extracts prepared from pVHL-defective renal carcinoma cells stably transfected to produce wild-type pVHL or with empty vector. (B) shows *in vitro* degradation of ³⁵S-labeled wild-type, Leu562Ala, and Pro564Ala in xenopus egg extracts. (C) is an anti-HA immunoblot analysis of COS7 cells transiently transfected with 1.5 or 3.5 μ g of plasmids encoding wild-type or P564A HA-HIF1 α in the absence or presence of desferoxamine.

FIG 6 depicts proline hydroxylation linked to pVHL-binding. (A) is a MALDI-TOF analysis of wild-type, Pro564Ala, and Leu562Ala biotinylated HIF(556-575) peptides following incubation with rabbit reticulocyte lysate. (B) shows Gal4-HA-HIF (555-575) translated *in vitro* in the presence of 3H-Proline with rabbit reticulocyte lysate or wheat

germ extract and gel-band purified. Following acid hydrolysis proline and hydroxyproline were separated using thin layer chromatography. The dashed circle indicates positions of ninhydrin stained proline and hydroxyproline markers.

FIG 7 illustrates that pVHL specifically recognizes HIF1 α with hydroxylated proline 564. (A and B) shows binding of ^{35}S -labeled pVHL to biotinylated HIF1 α (556-575) peptides with the indicated substitutions of residues 561-568. (C) shows ts20 cells stably transfected to produce HA-pVHL grown at restrictive (lane 1) or permissive (lanes 2-6) temperature and immunoprecipitated anti-HIF1 α (lane 1 and 2) or anti-HA antibody (lanes 3-7). Bound proteins were eluted by boiling in sample buffer (lane 1 and 2) or treatment with the indicated peptides and then immunoblotted with anti-HIF1 α antibody. (D) shows pVHL-defective renal carcinoma cells stably transfected to produce wild-type pVHL (WT8) or with empty vector (RC3) metabolically labeled with ^{35}S methionine, lysed, and incubated with immobilized biotinylated HIF1 α (556-575) peptides with the indicated substitutions of residue 564. Specifically bound proteins were detected by autoradiography.

FIG 8 illustrates the production of TET r -cyclins A and E. (A) is a schematic of TET r -cyclin fusion protein. (B) shows production of TET r - cyclin A and TET r -cyclin E. Cells transfected to produce the indicated cyclin A or cyclin E proteins were lysed and immunoblotted (IB) with the indicated antibodies. (C) shows phosphorylation of pRB by TET r -cyclins A and E in SAOS-2 cells. pRB-defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated cyclins were lysed and immunoblotted with anti-HA antibody. The % of transfected cells in G1 and S phase was determined by fluorescence activated cell sorting (FACS).

FIG 9 shows DNA bound cyclins A and E differentially affecting transcription. (A) is a schematic of reporter plasmid (pUHC 13-3) that contains seven TET r operator sequences (TET o) upstream of a minimal CMV promoter that includes a TATA box. (B, C) show U2OS cells were cotransfected with plasmids encoding the indicated TET r fusion proteins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β -galactosidase. Numbers shown at the bottom of the graph indicate the amount of TET r plasmid (in μg). 48 hours later luciferase activity, normalized for β -galactosidase, was determined. Fold repression is the corrected luciferase value for TET r alone divided by the corrected luciferase values for the indicated TET r fusion proteins. Fold activation represents the corrected luciferase values for the indicated TET r fusion proteins divided by the corrected luciferase value for TET r alone.

FIG 10 illustrates transcriptional regulation by cyclins A and E dependent upon DNA binding. (A) shows U2OS cells cotransfected with plasmids encoding the indicated TET_r fusion proteins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β -galactosidase. 24 hours later doxycycline was added to a final concentration of 2 μ g/ml where indicated by a '+'. 24 hours later, luciferase activity, corrected for β -galactosidase activity, was determined and expressed as fold repression or activation relative to cells producing TET_r alone: (B) shows U2OS cells cotransfected with plasmids encoding the indicated cyclins along with the pUHC 13-3 reporter plasmid and a plasmid encoding β -galactosidase. Fold repression and activation was determined as in (A). (C) shows U2OS cells cotransfected with plasmids encoding TET_r or TET_r-cyclin E, along with a minimal HSV-TK promoter reporter plasmid containing the indicated number of TET_o binding sites and a plasmid encoding β -galactosidase. Doxycycline was added as in (A).

FIG 11 illustrates that cyclin box is required for transcriptional repression by DNA bound cyclin A. (A) U2OS cells were cotransfected with plasmids encoding the indicated TET_r-cyclin A variants along with the pUHC 13-3 reporter plasmid and a plasmid encoding β -galactosidase. Cell extracts were prepared and luciferase activity, corrected for β -galactosidase activity, was expressed as fold repression relative to cells producing TET_r alone. (B) The indicated TET_r-cyclin A variants were translated *in vitro* in the presence of ³⁵S-methionine and incubated with GST-cdk2 and glutathione Sepharose. Specifically bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. In parallel, 20% of the input proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. (c) pRB defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated TET_r-cyclin A variants were lysed and immunoblotted with anti-HA antibody.

FIG 12 shows transcriptional activation by cyclin E linked to its ability to bind to cdk2 and interact with substrates. (A) U2OS cells were cotransfected with plasmids encoding the indicated TET_r-cyclin E variants along with the pUHC 13-3 reporter plasmid and a plasmid encoding β -galactosidase. Cell extracts were prepared, and luciferase activity, corrected for β -galactosidase activity, was expressed as fold activation relative to cells producing TET_r alone. (B) The indicated TET_r-cyclin E variants were translated *in vitro* in the presence of ³⁵S-methionine and incubated with GST-cdk2 and glutathione Sepharose. Specifically bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. In parallel, 20% of the input proteins

were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. (c) pRB defective SAOS-2 cells transfected so as to produce HA-tagged pRB along with the indicated TETr-cyclin E variants were lysed and immunoblotted with anti-HA antibody.

FIG 13 shows transcriptional activation by DNA bound cyclin E dependent on cdk2 catalytic activity. (A, B) U2OS cells were transiently cotransfected with plasmids encoding TETr-cyclin A or E and, where indicated, increasing amounts of a plasmid encoding a dominant-negative (dn) form of cdk2. Cell extracts were prepared and luciferase activity, corrected for β -galactosidase activity, was determined. Corrected luciferase values were expressed as fold repression (A) or activation (B) relative to TETr alone. (C) U2OS cells were transiently transfected with plasmids encoding TETr-cdk2 or TETr-cdk2 (N132A) and a plasmid encoding either cyclin A or E. Cell extracts were prepared and luciferase activity, corrected for β -galactosidase activity, was determined. Corrected luciferase values were expressed as fold activation relative to TETr alone.

FIG 14 shows transcriptional effects mediated by cell-cycle dependent changes in endogenous cyclins E and A. (A,B) 3T3 cells stably transfected with a luciferase reporter plasmid containing 7 TETo sites (pUHC13-3) and a plasmid encoding TETr-cdk2 were serum-starved for 72 hours and subsequently re-fed with serum. At various timepoints thereafter aliquots of cells were removed and either lysed for immunoblot analysis with the indicated antibodies or analyzed for DNA content by propidium iodide staining followed by fluorescence activated cell sorting (FACS). (C) 3T3 cells stably transfected with a luciferase reporter plasmid containing 7 TETo sites (pUHC13-3) in the absence (open circles) or presence of a plasmid encoding TETr-cdk2 (closed squares) or TETr-cdk2 (N132A) (open squares) were serum-starved for 72 hours and then re-fed with serum in the presence or absence of doxycycline. At various timepoints thereafter luciferase assays were performed. To correct for general effects due to serum, the luciferase values at each timepoint in the absence of doxycycline were corrected by subtracting the luciferase assay obtained in the presence of doxycycline. After correction, the luciferase values for the two cell populations were expressed relative to the corresponding luciferase values obtained at time 0. In parallel, the cells producing TETr-cdk2 were lysed and immunoprecipitated with anti-cyclin A or anti-cyclin E antibodies. The immunoprecipitates were then used to phosphorylate Histone H1 *in vitro*.

EXAMPLES

Example 1. Characterization of Hypoxia-Responsive Polypeptides

In order to demonstrate the efficacy of the First embodiment of the invention, *e.g.* employing a hypoxia-responsive LGP, the interaction of pVHL and HIF was examined. A HIF1 α polypeptide that is sufficient to bind pVHL is disclosed herein. pVHL binds directly to a region of HIF1 α called the oxygen-dependent degradation domain (ODD). pVHL recognizes HIF produced in rabbit reticulocyte lysate but not HIF produced in wheat germ extracts or in *E. Coli*. Furthermore, wheat germ or *E. Coli*-derived HIF binds to pVHL following preincubation with a human, rabbit, or xenopus cell extracts at 37°C. For example, glutathione S-transferase-ODD fusion proteins produced in *E. Coli* were not recognized by VBC in farwestern assays (FIG. 3C). These proteins were recognized, however, after pre-incubation with a rabbit reticulocyte lysate. Similar results were obtained with GST-ODD fusion proteins of various sizes, thus excluding the possibility that the farwestern blot signal represents a spurious interaction between VBC and a reticulocyte-derived protein. VBC did not recognize GST-ODD fusion proteins incubated with a heat-inactivated reticulocyte lysate (FIG. 3D). Gal4-HIF fusion proteins containing HIF residues 555-575 bound specifically to immobilized GST-VHL, elongin B, elongin C complexes (FIG. 4A). Coupled *in vitro* transcription/translation of ³⁵S-labeled proteins was conducted according to the manufacturer's instructions (TNT, Promega). Also, a biotinylated peptide corresponding to HIF residues 556-575 bound to pVHL following pre-incubation with reticulocyte lysate (FIG. 4B). For peptide binding studies, 1 μ g of biotinylated peptide was bound to 30 μ l of monomeric avidin Sepharose (Pierce). Where indicated, the peptide was pre-incubated with 50 μ l of rabbit reticulocyte lysate for 90 min at 30°C. The Sepharose was then washed 3 times with NETN (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% non-idet P40) and used in binding reactions containing 4 μ l ³⁵S-HA-pVHL in 500 μ l of EBC or 500 μ l ³⁵S-radiolabeled cell extract (equivalent to cells from a subconfluent 100mm dish). Following 1 hour incubation at 4°C with rocking the Sepharose was washed 4 times with NETN. Bound proteins were eluted by boiling in SDS-containing sample buffer and detected by autoradiography. This region of HIF contains a highly conserved 8 mer (MLAPYIPM) which, when mutated to 8 consecutive alanines, leads to HIF stabilization in cells. An alanine scan of this region showed that Leu562 and Pro564 were essential for specific binding to pVHL in this assay (FIG. 4B). In contrast, mutation of the one potential phosphoacceptor in this peptide, Tyr565, did not affect pVHL binding, in

keeping with an earlier study in which a Tyr565Phe mutation did not affect HIF stability. In addition, the binding of pVHL to GST-ODD in the assays described above was unaffected by phosphatase treatment.

Binding of pVHL to HIF1 α is critically dependent upon residues Leu562 or Pro564 of HIF1 α . Importantly, mutation of either Leu562 or Pro564 in the context of full-length HIF1 α or a Gal4-ODD fusion protein also led to a loss of pVHL binding activity (FIG. 4C and 4D, respectively). Gal4-ODD made with reticulocyte lysate contained an electrophoretically distinct band compared with Gal4-ODD made with wheat germ extract (FIG. 4D). This electrophoretically distinct protein bound to VBC and was undetectable among the Leu562Ala and Pro564Ala translation products. The isoelectric points of the two arrowed bands in FIG 4D were identical following 2-D gel electrophoresis indicating that the putative modification did not involve a change in protein charge.

Modification of HIF1 α by pVHL following binding is also critically dependent upon residues Leu562 or Pro564 of HIF1 α . Gal4-HIF fusion proteins with the Leu562Ala mutation or Pro564Ala mutation displayed diminished pVHL-dependent polyubiquitination *in vitro* relative to the corresponding wild-type protein (FIG. 5A). Qualitatively similar results were obtained with the corresponding full-length HIF1 α species, although this assay is less robust than with the Gal4-ODD fusion proteins. Likewise, HIF1 α Pro564Ala and HIF1 α Leu562Ala were far more stable than wild-type HIF1 α in *in vitro* degradation assays performed with xenopus extracts (FIG. 5B). Xenopus egg extracts were made as is well known in the art and stored frozen until use. Degradation reactions contained 8 μ l of egg extract, 0.1 μ l of 100 mg/ml cyclohexamide, 0.25 μ l of energy regeneration mix, 0.25 μ l of bovine ubiquitin, and 0.4 μ l of ³⁵S-radiolabeled HIF and were carried out at room temperature. At the indicated timepoints 1 μ l aliquots were removed and placed in sample buffer. Samples were resolved on 5-15% gradient gels and analyzed by autoradiography. Biotinylated HIF (556-575) peptides were incubated with rabbit reticulocyte lysate, washed, eluted with free biotin, and analyzed by mass spectrometry. 1 μ g of HPLC-purified peptide was bound to 30 μ l monomeric avidin Sepharose and incubated with 100 μ l of rabbit reticulocyte lysate at room temperature for 1 hour with tumbling. Following brief centrifugation the reticulocyte lysate was removed, fresh reticulocyte lysate was added, and the cycle was repeated 6 times. The Sepharose was then washed 4 times with NETN and once with PBS. The modified peptide was eluted in 50 μ l of 20 mM ammonium acetate [pH 7.0], 2 mM biotin. In these experiments Met561 and Met568 were converted to alanine

to prevent spurious oxidation of the methionine residues during analysis. This double alanine substitution, like the corresponding single substitutions, did not affect pVHL binding. Treatment of the HIF (556-575) peptide with rabbit reticulocyte lysate led to the appearance of a second peak in MALDI-TOF assays representing an increase in molecular weight of 16 (FIG. 6A). This peak was not detectable prior to incubation with reticulocyte lysate and was not detected in the corresponding reticulocyte-treated Leu562Ala and Pro564Ala peptides (FIG. 6A). Postsource decay analysis using the same instrument placed the addition of +16 at Pro 564. Similarly, electrospray ion trap mass spectrometry/mass spectrometry (MS/MS) analysis was consistent with addition of +16 at Pro 564 and excluded such a modification of Leu 562. Finally, MS/MS analysis of the reticulocyte-treated HIF (556-575) peptide produced a pattern of ions that was identical to the pattern obtained with a HIF (556-575) peptide which was synthesized to contain hydroxyproline at position 564. Next, Gal4-HIF(555-575) was translated in vitro in the presence of ^3H -Proline using rabbit reticulocyte lysate or wheat germ extract, gel-band purified, and subjected to acid hydrolysis and thin layer chromatography. 2 ml of ^3H -P-labeled Gal4-HIF (555-575) in vitro translate was immunoprecipitated with 50 μg of anti-HA antibody (12CA5, Roche), resolved on a 12% SDS-polyacrylamide gel, and transferred to a PVDF membrane. Gal4-HIF (555-575) was visualized by autoradiography and the corresponding region of PVDF was excised, hydrolyzed by incubation in 100 μl of 10 N HCl at 105°C for 3 hours. Samples were evaporated to dryness, resuspended in 20 μl H_2O containing 10 μg of unlabeled proline and 4-OH proline (Sigma), and resolved by 2-D thin layer chromatography using phenol-distilled H_2O in the first dimension and N-butanol-acetic acid- H_2O in the second. Following visualization of standards with ninhydrin radiolabeled proline was detected by autoradiography. Gal4-HIF produced in rabbit reticulocyte lysate, but not in wheat germ, contained hydroxyproline (FIG. 6B).

pVHL specifically recognizes a proline hydroxylated determinant. The HIF (556-575) peptide containing hydroxyproline at position 564 bound to pVHL with or without pretreatment with reticulocyte lysate (FIG. 7A). The mass spectrometry analysis of the Leu562Ala peptide showed that Leu562 was required for HIF modification (FIG. 6A). Indeed, pVHL bound to a HIF (556-575) peptide with the Leu562Ala substitution and hydroxyproline at residue 564 (FIG. 7B). This suggests that the primary role of Leu562 is to allow for the hydroxylation of Pro 564. Two approaches were used to demonstrate that hydroxylated HIF peptide could interact with cell-derived pVHL complexes. Ts20 cells

were engineered to produce HA-tagged pVHL which carry a temperature-sensitive mutation in the E1-ubiquitin activating enzyme. ts20 cells were transfected with pIRES-HA-VHL, pIRES-HA-VHL (Y98H), or pIRES-Neo (Invitrogen) and selected in the presence of 1mg/ml G418. Individual G418-resistant colonies were isolated using cloning cylinders and expanded. Cells producing HA-VHL or HA-VHL (Y98H) were identified by anti-HA immunoblot analysis. HIF coimmunoprecipitated with HA-pVHL at the restrictive temperature. HIF bound to pVHL in this way could be eluted by the hydroxylated HIF (556-575) peptide but not the unmodified peptide (FIG. 7C). For the tests shown in FIG. 7C, ts20 cells were grown at the restrictive or permissive temperature for 14 hours, methionine-starved for 90 min, and then grown in the methionine-free media supplemented with ³⁵S-met (500 mCi/ml) for 90 min. Cells were washed once with cold PBS, lysed in EBC, and immunoprecipitated with anti-HA (12CA5; Roche or anti-HIF1 α (NB100-105; Novus). Following 5 washes with NETN bound proteins were eluted by boiling in sample buffer or by incubation in 65 μ l of PBS containing 7 μ g of the indicated peptide. Moreover, HIF was not eluted by the HIF (556-575) Pro564Ala peptide or by a poly-hydroxyproline peptide (FIG. 7C). Affinity chromatography was performed with immobilized peptides and metabolically labeled matched renal carcinoma cells which do (WT8) or do not (RC3) produce HA-pVHL. 786-O subclones were starved for 1 hour, grown in the methionine-free media supplemented with ³⁵S-met (500 mCi/ml) for 3 hr, washed once with ice cold PBS, and lysed in EBC. The proline hydroxylated HIF (556-575) peptide specifically bound to pVHL as well as proteins with the expected electrophoretic mobilities of the pVHL-associated proteins elongin B, elongin C, and Cul2 (FIG. 7D). The identity of pVHL in this experiment was confirmed by western blot analysis. Likewise, the binding of endogenous pVHL to the hydroxylated peptide was detected using 293 embryonic kidney cells. A HIF1 α mutant in which Proline 564 was converted to alanine was stabilized in cells and insensitive to the hypoxia-mimetic desferoxamine (FIG. 5C).

The HIF1 α protein is stabilized in hypoxic conditions and functions to inhibit, decrease and/or reverse hypoxia in affected tissues, *e.g.* in a solid tumor, diabetic retinopathy or ischemic heart tissue, in part by modulating pro-angiogenic factors. A light-generating fusion protein containing a HIF1 α moiety will also be stabilized in hypoxic tissue. Destruction of a HIF1 α protein or a HIF1 α -containing LGP via the pVHL ubiquitin pathway occurs, *e.g.* during normoxia, when a prolyl hydroxylase modifies the HIF1 α protein such that pVHL binds to and modifies HIF1 α . This modification process acts as a

dynamic switch to regulate the bioluminescence of the HIF1 α -containing light-generating fusion protein such as the First embodiment of the invention. A HIF1 α -containing light-generating fusion protein is useful to dynamically image hypoxic tissues, e.g. cancer, *in situ*, and to screen for or test the efficacy of hypoxia-modulating compounds.

5 **Example 2**

The following example demonstrates the efficacy of light-generating fusion proteins of the invention for imaging hypoxic tissues.

The Oxygen-Dependent Degradation Domain (ODD) of HIF1 α , renders HIF1 α unstable in the presence of oxygen. This region is recognized by pVHL. pVHL
10 specifically binds directly to peptidic determinants, corresponding to HIF1 α residues 555-575, located within the ODD. At the core of this peptide there is a conserved proline residue (residue 564) which, in the presence of oxygen, becomes enzymatically hydroxylated. This residue serves as the signal for pVHL to bind. In sum, in the presence of oxygen, the HIF peptide becomes hydroxylated and is recognized by pVHL. In the
15 absence of oxygen (hypoxia), the modification does not take place and pVHL does not bind to HIF.

Construction of ODD-LUC plasmids

In a first set of experiments the ability of a small HIF peptide (555-575) to function *in cis* to target a foreign protein for pVHL-dependent proteolysis was evaluated. To this
20 end, the HIF cDNA encoding amino acids 555- 575 (hereafter 'ODD') was PCR amplified with oligonucleotides that introduced convenient restriction sites, digested, and gel-band purified. The PCR fragment was then subcloned, in-frame, 3' of a Firefly luciferase cDNA contained in the pGL3 plasmid (Promega). The resulting luciferase-ODD chimeric cDNA was subcloned into pcDNA3 (Invitrogen) to facilitate *in vitro* translation and expression
25 studies in mammalian cells. In parallel a pcDNA3 plasmid encoding wild-type Firefly luciferase and pcDNA3 plasmid encoding wild-type HIF1 α was used as controls.

VBC-GST Pulldown of ODD-Luciferase

To determine whether the ODD-luciferase protein could bind to pVHL, GST-VHL, elongin B, and elongin C ('GST-VBC') were produced in E.Coli and recovered as a
30 trimeric complex on glutathione sepharose. Earlier work showed that pVHL does not fold properly in the absence of elongin B and elongin C. HIF1 α , ODD-luciferase, and wild-type luciferase were translated *in vitro* using rabbit reticulocyte lysate (RRL) or wheat germ extract (WG) in the presence of ³⁵S methionine (FIG 15). Aliquots from the *in vitro*

translation reactions were added to recombinant VBC-GST prebound to glutathione sepharose and incubated for 1 hour. The sepharose was then washed and bound proteins were resolved on a 12% SDS polyacrylamide gel. The gel was then dried and exposed to film. pVHL bound to reticulocyte-generated HIF1 α and ODD-Luc, but not Luc (compare lanes 4, 8 and, 12). Furthermore, pVHL did not bind to wheat germ produced ODD-Luc, as wheat germ lacks the HIF prolyl hydroxylase (compare lanes 6 and 8). Thus the data show that ODD-Luc was specifically recognized by pVHL.

Peptide Competition Assay

The observation that pVHL bound to ODD-Luc produced in RRL, but not WG, strongly suggested that the ODD-Luc, like HIF, needed to undergo prolyl hydroxylation in order to be recognized by pVHL. To study this further, the GST-VBC binding experiments were repeated in the presence of synthetic HIF (555-575) peptides in which Pro 564 was hydroxylated (P-OH) or was not hydroxylated (P). GST-VBC was mixed with either the control (P) peptide or the P-OH peptide in increasing concentrations (from 0 – 5 μ g). Next *in vitro* translated (in retic lysate) Luc, ODD-Luc, or HIF1 α was then added to see if it could efficiently compete off any bound P-OH. HIF and ODD-Luc were able to compete off the P-OH peptide effectively at even very high concentrations of P-OH. Thus, the results obtained with ODD-Luc recapitulated earlier binding studies performed with authentic HIF. (FIG-16.)

Luciferase Activity in Cells

In pilot experiments, it was confirmed that ODD-Luc *in vitro* translate retained luciferase activity *in vitro* comparable to wild-type luciferase. To begin to ask whether the ODD-Luc was oxygen sensitive in cells, transient transfection assays were performed. In the first set of experiments, 100 mm tissue culture plates were seeded with 8×10^5 HeLa cells per plate. Eighteen hours later the cells were transiently transfected, using lipofectamine (Gibco), with the pcDNA3 plasmids encoding ODD-Luc or wild-type Luc along with an internal control plasmid encoding Renilla luciferase. Twenty-four hours after transfection the cells were split into 6 well plates and allowed to adhere and grow for 8-12 hours. At this point the hypoxia mimetics desferrioxamine (DFO) or cobalt chloride (CoCl₂) were added directly to the media at final concentrations of 500 mM and 200 mM respectively, in duplicate wells. In addition, some wells were not treated so as to serve as controls. 12 hours after treatment with DFO and CoCl₂ the cells were lysed with Passive Lysis Buffer (Promega), rocked at room temperature for 20 minutes, and assayed for Firefly and Renilla

Luciferase according to the manufacturers instructions. Results were obtained in duplicate and averaged. (FIG 17)

In the untreated cells the luciferase values for ODD-Luc were approximately 30% of the values obtained with wild-type Luc. Note that HeLa cells have wild-type pVHL and these experiments were conducted using cells grown in the presence of oxygen. The addition of hypoxia-mimetics led to a marked increased in ODD-Luc luciferase activity, whereas no such induction was detected with wild-type luciferase. The induced levels of ODD-luciferase were comparable to those obtained with wild-type luciferase. These results are consistent with the idea that ODD-Luc is subject to pVHL-dependent proteolysis in cells whereas wild-type Luc is not.

Imaging of ODD-Luc in Xenograft Tumors in Nude Mice.

To verify in a mammalian system that the ODD-Luc gene is indeed regulated by hypoxia or hypoxia-mimetics, the following experiment was conducted. Given the ease of subcutaneously transplanting tumors and their ability to grow and vascularize, the ability to image luciferase activity of tumors at the subcutaneous level and assess ODD-Luc's response to hypoxia was tested as follows.

Polyclonal HeLa cells stably transfected with ODD-Luc and Luc were generated. The clones were suspended in PBS and counted. 10^6 cells per injection site were administered subcutaneously in duplicate into the flanks of nude mice (FIG 18). Upon growth of palpable tumors (approximately 3-4 days) the mice were given intraperitoneal injections of phenobarbital for anesthesia, injected with a weight-adjusted dose of luciferin, and whole body imaged with a Xenogen Camera. To ensure that the difference in luciferase activity was not simply a function of tumor size alone, bidimensional tumor measurements were taken and were approximately equal.

FIG 18 shows three different mice injected with ODD-Luc (right) and Luc (left) in duplicate. The sites where ODD-Luc was injected clearly have attenuated luciferase signal. As such, the data show a real attenuation of luciferase activity, secondary ubiquitination and destruction of ODD-Luc.

Example 3. Light-Generating Fusion Proteins Including a DNA Binding Site

In order to demonstrate the efficacy of the Second embodiment of the invention, a LGP capable of interacting with nucleic acids in order to kinetically monitor gene transcription *in vivo*, *in vitro* or *in silico*, the impact of cyclins on transcription was
5 examined. As it is useful to monitor either the induction or repression of transcription, cyclins with specific affects on transcription were investigated.

Fusion of a DNA-binding motif to a cyclin does not alter cyclin binding to a cdk or the kinase activity of the cyclin/cdk complex. Mammalian expression plasmids were generated that encode fusion proteins consisting of the TET repressor DNA-binding domain
10 (TETr) (Gossen and Bujard, 1992) fused to cyclin A or cyclin E with an intervening flexible linker consisting of Gly4-Ser repeats (FIG 8A). Both of these plasmids gave rise to stable proteins of the expected size following transfection into mammalian cells (FIG 8B). TETr-cyclin A and TETr-cyclin E, like their unfused counterparts, bound to cdk2 (FIG 11 and 12) and could phosphorylate p107 *in vitro*. Furthermore, both TETr-cyclin A and TETr-cyclin
15 E promoted pRB phosphorylation and bypassed a pRB-induced G1/S block when cointroduced with wild-type pRB into pRB-defective tumor cells (FIG 8C).

Cyclin A and cyclin E dramatically affect transcription. U2OS cells were transiently transfected with plasmids encoding various TETr fusion proteins and a luciferase reporter plasmid containing 7 TETo binding sites upstream of a TATA box
20 derived from the CMV promoter (FIG. 9A). TETr binds specifically to TETo sites. As expected, TETr-RB repressed transcription from this reporter plasmid whereas TETr-E2F1 activated the reporter (FIG 9B-C). The basal activity observed with this reporter plasmid presumably reflects the presence of cryptic enhancer sequences. In this and subsequent assays, the TETr domain alone was essentially inert. Surprisingly, TETr-cyclin A and
25 TETr-cyclin E both dramatically affected transcription in this assay and did so in opposite ways. TETr-cyclin A decreased transcription approximately 80% (5-fold repression) whereas TETr-cyclin E increased transcription 10-fold (FIG. 9B-C). Doxycycline prevents the binding of TETr to TETo and completely blocked the transcriptional effects of TETr-cyclin A and TETr-cyclin E (FIG 10A). As expected, doxycycline also blocked the
30 transcriptional effects of TETr-RB and TETr-E2F1, which were tested in parallel. Furthermore, unfused cyclin A and E had no effects on the TETo-driven reporter plasmid (FIG 10B). Experiments were repeated using reporters containing 1, 2, 3, or 7 TETo in which the CMV-derived TATA box was replaced with a minimal HSV TK promoter

(Gossen and Bujard, 1992) (FIG 10C). TET_r-cyclin E also activated these reporters in doxycycline-inhibitable manner. The degree of activation observed with the HSV TK series of reporters was lower than with the CMV TATA-based reporter, in keeping with earlier results obtained with these reporters and fused TET_r to the HSV VP16

transcriptional activation domain (Gossen and Bujard). The low basal level of transcription from these reporters precluded analysis of repression by cyclin A.

The specific domains of the cyclins that bind to the cdk2s are critical for cyclin-mediated transcriptional regulation. Plasmids encoding TET_r fused to various colinear fragments of cyclin A and E were used to determine which regions of these molecules are required for transcriptional regulation (FIG 11 and 12). All of the resulting fusion proteins were expressed at comparable levels in transient transfection experiments. Cyclin A (1-310), like wild-type cyclin A, repressed transcription when fused to TET_r (FIG 11A). This fragment of cyclin A does not bind to cdk2 (FIG 11B) and cannot direct the phosphorylation of pRB when introduced into cells (FIG 11C). Conversely, a cyclin A point mutant (E220A) (Schulman *et al.*, 1998) that measurably interacts with cdk2 (FIG 11B) and directs the phosphorylation of pRB (FIG 11C) did not repress transcription in these assays (FIG 11A). This mutation maps to the cyclin A cyclin box (FIG 11A). TET_r-cyclin A also repressed transcription when tested in p107^{-/-};p130^{-/-} mouse fibroblasts and cyclin A (1-310) does not bind to either p107 or p130. Only those cyclin E mutants that bind to cdk2 (FIG 12B) and could direct the phosphorylation of pRB (FIG 12C) scored as transcriptional activators (FIG 12A). For example, Schulman *et al* (1998) identified cyclin A residues that are critical for substrate binding and assembly with cdk2. Mutation of analogous residues in cyclin E produced a mutant (cyclin E L134A/Q174A) that likewise failed to bind to cdk2 (FIG 12B) and failed to phosphorylate pRB (FIG 12C). This mutant did not activate transcription (FIG 12). In keeping with these results, a dominant-negative form of cdk2 blocked transcriptional activation by cyclin E (FIG 13B) but had no effect on transcriptional repression by cyclin A (FIG 13A). Similarly, cyclin E, but not cyclin A, activated transcription in concert with a TET_r-cdk2 fusion provided the kinase domain was intact (FIG 13C). Comparable production of TET_r-cdk2 and kinase-defective TET_r-cdk2 (N132A) was confirmed by immunoblot assay. *Xenopus* cyclin E (Jackson *et al.*, 1995), like its human counterpart, also activated transcription in these assays (data not shown). This activity was specific as *xenopus* cyclin E variants with point mutations affecting the cyclin box were inert.

The Second embodiment of the invention is useful to dynamically image transcription *in vivo*. For example, cyclin E can activate transcription under physiological conditions. 3T3 cells were transfected with the plasmid containing a selectable marker and TETo reporter plasmid with or without a plasmid encoding TETr-cdk2. Following drug
5 selection, the stable transfectants were maintained as polyclonal pools and serum starved into quiescence. At various timepoints after serum refeeding, cell lysates were prepared and used in immunoblot, *in vitro* kinase, and luciferase assays (FIG 14). In parallel, aliquots of the cells were analyzed for DNA content by FACS. In this system, S-phase entry began 18-
20 hours following the addition of serum. As expected, luciferase activity increased in the
10 TETr-cdk2 producing cells coincident with an increase in cyclin E protein levels and cyclin E-associated kinase activity (FIG 14A-C). No such increase was observed in the cells producing equivalent amounts of TETr-cdk2 (N132A) or transfected with the reporter alone (FIG 14C and data not shown). Note that the amount of TETr-cdk2 in these cells was less than the amount of endogenous cdk2 (FIG 14B). Thus, the results are unlikely to be an
15 artifact of overproduction. Luciferase values declined as cyclin E levels fell and cyclin A levels began to rise.

The Second embodiment of the invention in part relates to LGPs containing cyclin-binding domains. Thus, these LGPs are useful to dynamically quantify alterations in transcription of cell-cycle associated genes, such as oncogenes and tumor suppressors. A
20 LGP containing a cyclin-binding moiety can be localized to regions undergoing cell proliferation, such as a tumor, and can be used to screen for and determine the efficacy of cell proliferation-modulating compounds.

MATERIALS AND METHODS

Cell lines and transfection

25 U2OS human osteosarcoma cells were grown in Dulbecco's modified Eagle media (DMEM) supplemented with 10% heat-inactivated fetalclone (Hyclone) (FC), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (PSG). SAOS-2 human osteosarcoma cells and NIH 3T3 mouse fibroblast cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and PSG. NIH 3T3
30 stable subclones transfected with the pCMV-neo and pUHC13-3 reporter plasmid alone or with pSG5-TETr-cdk2 or with pSG5-TETr-cdk2(N132A) were maintained in 0.7 mg/ml of G418. Cells were transfected using 2x Bes-buffered saline (2xBBS)/calcium phosphate as described (Chen and Okayama, 1987). Where indicated doxycycline (Sigma) was added 24

hours after transfection to a final concentration of 2 µg/ml. Cells were maintained in doxycycline for an additional 24 hours prior to harvest.

Plasmids

pRcCMV-cdk2 dominant negative (van den Heuvel and Harlow, 1993) was a gift of Dr. Ed Harlow; pVL1393-cdk2(N132A) (Xu et al., 1994) was a gift of Dr. Helen Piwnicka-Worms; pCD19 (Tedder and Isaacs, 1989) was a gift of Dr. Thomas Tedder and pUHC13-3; ptet1-T81-luc, ptet2-T81-luc, ptet3-T81-luc, and ptet7-T81-luc (Gossen and Bujard, 1992) were gifts of Dr. Manfred Gossen. pSG5-TETTr-E2F1, pSG5-TETTr-RB, pSG5-HA-RB (Sellers, 1995), pGEX-2TK-cdk2 (Adams *et al.*, 1996) have been described previously.

To make pSG5-TETTr-cyclin A, a protein phosphatase 1 (PP1) cDNA was first PCR amplified with oligos 5'-GCGCTGATCAGGCGGAGGCGGATCAGGAGGAGGAGGATCAGGCGGAGGAGGA TCAGGATCCATGTCCGACAGCGAGAA-3' (SEQ ID NO: 7) and 5'-GCGCGAATTCATTTCTTGGCTTTGGCAGA-3' (SEQ ID NO: 8). The PCR product was cut with BclII and EcoRI and subcloned into pSG5-TETTr cut with BamHI and EcoRI to make pSG5-TETTr- (Gly4-Ser)3-PP1. The cyclin A open reading frame (ORF) was PCR amplified with primers that introduced a 5' BamHI site and a 3' EcoRI site and subcloned into pSP72 (Promega) cut with these two enzymes to make pSP72-cyclin A. The PP1 'stuffer' from pSG5-TETTr- (Gly4-Ser)3-PP1 was then excised by digestion with BamHI and EcoRI and replaced with the cyclin A cDNA insert from pSP72-cyclin A. To make pSG5-TETTr-cyclin E, the cyclin E ORF in pRcCMV-cyclin E was PCR amplified with primers that introduced a 5' BglII and 3' EcoRI site. The PCR product was cut with these two enzymes and ligated into the BamHI-EcoRI backbone of pSG5-TETTr-PP1. In parallel, these restricted cyclin A and cyclin E PCR products were subcloned into pSG5-HA cut with BamHI and EcoRI to make pSG5-HA-cyclin A and pSG5-HA-cyclin E, respectively.

Plasmids encoding cyclin A and cyclin E N-terminal and C-terminal deletion mutants were made in an analogous fashion by using PCR primers that selectively amplified the desired coding regions. To make pSG5-TETTr-cdk2 and pSG5-TETTr-cdk2 (N132A), the cdk2 ORF in pRcCMV-cdk2 and pVL1393-cdk2 (N132A), respectively, were PCR amplified with primers that introduced a 5' BamHI and 3' EcoRI site. The PCR products were cut with these two enzymes and ligated into the BamHI-EcoRI backbone of pSG5-TETTr-PP1. All PCR reactions were performed with Pfu DNA polymerase and the authenticity of plasmids containing the entire cyclin A, cyclin E, or cdk2 open reading frame was confirmed by direct DNA sequencing. pSG5-TETTr-cyclin A (E220A) and pSG5-TETTr-cyclin E

(L134A/Q174A) were generated using Transformer Site-Directed Mutagenesis Kit (Clontech) according to manufacture's instructions using pSG5-TET_r-cyclin A and pSG5-TET_r-cyclin E as a template, respectively, and confirmed by DNA sequencing.

Antibodies and immunoblot analysis

5 Monoclonal anti-TET_r was purchased from Clontech and anti-HA (12CA5) was purchased from Boehringer Mannheim. Polyclonal anti-cyclin A (SC-751), monoclonal and polyclonal anti-cyclin E (SC-247, SC-481), and polyclonal anti-cdk2 (SC-163) were purchased from Santa Cruz. Cell extracts were made by lysis in EBC buffer (50mM Tris [pH8], 120mM NaCl, 0.5% Nonidet P-40). For immunoblot analysis, ~100 µg of cell
10 extract was loaded per lane. Nitrocellulose filters were blocked in 4% powdered milk/ 1% goat serum in TBS-T (10mM Tris [pH 8], 0.05% Tween, 150mM NaCl) for 1 hour at room temperature prior to incubation in primary antibody. Anti-HA (12CA5) was used at a concentration of 1.0 µg/ml, anti-TET_r antibody at 1:500 dilution (v/v), anti-cyclin A (SC-751) at 1:1,000 dilution (v/v), anti-cyclin E (SC-247, SC-481) at 1:1,000 dilution (v/v) and
15 anti-cdk2 (SC-163) at 1: 1,000 dilution (v/v). Following 4 washes with TBS/T, bound antibody was detected using alkaline phosphatase-conjugated secondary antibodies.

GST pull-down assay

 Glutathione S-transferase pull-down assays were performed basically as described previously (Kaelin *et al.*, 1991). Binding reactions contained 10 µl of ³⁵S-radiolabelled in
20 vitro translates made with a TNT kit (Promega) and approximately 1 µg of the indicated GST fusion protein in 1ml of NETN (20mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Following 1 hour incubation at 4°C with rocking, the Sepharose was washed 5 times with NETN. Bound proteins were eluted by boiling in SDS-containing sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Comparable
25 loading of GST-fusion proteins was confirmed by Coomassie brilliant blue staining and ³⁵S-radiolabelled proteins were detected by fluorography.

FACS/cell cycle analysis

 Fluorescence activated cell sorting (FACS) was done essentially as described (Qin *et al.*, 1995). Briefly, subconfluent SAOS-2 cells grown in 100 mm dishes were transfected
30 with 2 µg of pCD19 and 10 µg of pSG5-HA-RB together with plasmids encoding the indicated cyclins. 72 hr later the cells were harvested with trypsin-EDTA and stained with FITC-conjugated anti-CD19 antibody (CALTAG) and propidium iodide. Samples were analyzed by two-color FACS with a FACScan (Becton Dickinson). For cell cycle

synchronization, cells were starved in serum-free DMEM for 72 hours before being stimulated with 10% FBS.

Luciferase reporter gene assay

For TET^r-fusion transcriptional assay, subconfluent U2OS cells were transiently
5 transfected in 6-well plates in duplicate with 1 µg of pCMV-µgal, 1 µg of pUHC13-3
reporter plasmid, and 3 µg of the indicated plasmids encoding TET^r-fusion proteins.
Sufficient parental pSG5-TET^r was added so that each reaction mix contained the same
amount of pSG5-TET^r backbone. 48 hours after transfection luciferase activity and β-
galactosidase activity was determined as described previously (Qin, 1995).

10 ***In vitro kinase assay***

500 µg of cell extract was incubated with protein A Sepharose and 1 µg of anti-
cyclin E (SC-481) or anti-cyclin A (SC-751) antibody for 1 hour at 4°C in a final volume of
0.5 ml. The Sepharose was then washed 5 times with NETN and 3 times in IP kinase (IPK)
buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM DTT). The Sepharose was then
15 resuspended in 27 µl of IPK buffer to which was added 2 µl of histone H1 (1mg/ml) and 1
µl of [γ-³²P] ATP (6,000 Ci/mmol, 10 mCi/ml) and incubated for 30 min at 30°C.
Reactions were stopped by addition of Laemmli sample buffer, boiled, resolved by SDS-
polyacrylamide gel electrophoresis, and subjected to autoradiography.

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5 EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the present invention and are covered by the following claims. The contents of all references, issued patents, and
10 published patent applications cited throughout this application are hereby incorporated by reference. The appropriate components, processes, and methods of those patents, applications and other documents may be selected for the present invention and embodiments thereof.

CLAIMS

What is claimed is:

1. A method of identifying prolyl hydroxylase modulators, comprising the steps of:
 - a) contacting a prolyl hydroxylase, a putative prolyl hydroxylase modulator and a light-generating fusion protein comprising an HIF α polypeptide moiety having binding character for prolyl hydroxylase and a light-generating polypeptide moiety, wherein the light generation of said light-generating polypeptide moiety changes upon binding of a prolyl hydroxylase to said HIF α polypeptide moiety, under conditions favorable for binding of a prolyl hydroxylase to a HIF α to form a test sample; and
 - b) determining the ability of said putative prolyl hydroxylase modulator to modulate prolyl hydroxylase by measuring the light generated in said test sample.
2. An assay for a modulator of prolyl hydroxylase, comprising
 - a) bringing into contact a prolyl hydroxylase, a HIF1 α polypeptide moiety and a putative modulator compound under conditions where prolyl hydroxylase, in the absence of modulator, is capable of acting on said HIF1 α polypeptide moiety; and
 - b) measuring the degree of prolyl hydroxylase inhibition caused by said modulator compound.
3. The method of claim 2, wherein said test sample further comprises pVHL.
4. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises the amino acid sequence Y-X₁-Leu-X₂-Pro_h-X₃-X₄-X₅-X₆-Y', wherein
 - a) Pro_h is hydroxylated proline;
 - b) X₁, X₂, X₄, X₅, and X₆ are independently Gly, Ala, Val, Leu, Ile, Pro, Met, Phe, or Trp;
 - c) X₃ is Ser, Thr, or Tyr; and
 - d) Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids.

5. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
6. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 402 is hydroxylated proline.
7. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein either or both of residues 402 and 564 are hydroxylated proline.
8. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises a 4 to 12 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
9. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises a 12 to 14 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
10. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises a 20 to 30 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
11. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises a 80 to 120 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
12. The method of claim 1, whereupon ligand binding to said ligand binding site alters the light generation of said light-generating fusion protein without altering the phosphorylational state of said light-generating fusion protein.

13. The method of claim 2, wherein said HIF1 α polypeptide moiety comprises amino acids 555-575 of wild type HIF α , numbered in accordance with wild type HIF α .
14. HIF α polypeptide comprising amino acids 555-575 of wild type HIF α , numbered in accordance with wild type HIF α .
15. A method of treating or preventing a hypoxic or ischemic related disorder in a subject, comprising administering to a subject in need thereof a compound identified by the method of claim 1 which decreases prolyl hydroxylase expression or activity, such that said hypoxic or ischemic related disorder is treated.
16. The method of claim 15, wherein said compound is a prolyl hydroxylase antibody, or a nucleic acid that decreases the expression of a nucleic acid that encodes a prolyl hydroxylase polypeptide.
17. The method of claim 16, wherein the nucleic acid is a prolyl hydroxylase anti-sense nucleic acid.
18. The method of claim 15, wherein the hypoxic or ischemic related disorder is an acute event selected from the consisting of myocardial infarction, stroke, cancer, and diabetes.
19. The method of claim 15, wherein the hypoxic or ischemic related disorder is a chronic event not caused by tissue scarring.
20. The method of claim 15, wherein the hypoxic or ischemic related disorder is a chronic event selected from the group consisting of deep vein thrombosis, pulmonary embolus, and renal failure.
21. The method of claim 15, wherein the half life of HIF in said subject is increased compared to a subject not exposed to said compound.
22. A method of increasing angiogenesis or vascularization in a subject, comprising administering to a subject in need thereof a compound identified by the method of claim 1 which decreases prolyl hydroxylase expression or activity.
23. A method of treating cancer in a subject, comprising administering to a subject in need thereof a compound identified by the method of claim 1 which increases prolyl hydroxylase expression or activity.
24. A method of treating a cell-proliferating disorder by administering to a subject an effective amount of a fusion protein comprising a HIF α polypeptide moiety having a

- binding character for prolyl hydroxylase, and a suicide polypeptide moiety, such that said cell-proliferating disorder is treated.
25. A method of treating a hypoxic or ischemic disorder by administering to a subject an effective amount of a fusion protein comprising a HIF α polypeptide moiety having a binding character for prolyl hydroxylase, and a suicide polypeptide moiety, such that said hypoxic or ischemic disorder is treated.
26. A method of killing hypoxic tumor cells, comprising administration of an effective amount of a fusion protein to a subject, said fusion protein comprising a HIF α polypeptide moiety having a binding character for prolyl hydroxylase, and a suicide polypeptide moiety, such that said hypoxic tumor cells are killed.
27. A method of monitoring the treatment of a cell-proliferating disorder by
- a) administering to a subject an effective amount of a fusion protein comprising a HIF α polypeptide moiety having a binding character for prolyl hydroxylase, a suicide polypeptide moiety, and a light-generating polypeptide moiety, wherein the light generation of said light-generating fusion protein changes upon binding of prolyl hydroxylase to said HIF α polypeptide moiety, such that said cell-proliferating disorder is treated, and
 - b) monitoring the ability of said fusion protein to inhibit cell proliferation by measuring the light generated by said light-generating fusion protein.
28. A method of treating a cell-proliferating disorder by administering to a subject an effective amount of a fusion protein comprising a cyclin/cdk binding site and a suicide protein polypeptide moiety, such that said cell-proliferating disorder is treated.
29. A method of monitoring the treatment of a cell-proliferating disorder by
- a) administering to a subject an effective amount of a light-generating fusion protein comprising a cyclin/cdk binding site, a suicide protein polypeptide moiety, and a light-generating polypeptide moiety, wherein the light generation of said light-generating fusion protein changes upon binding of a cyclin to said cyclin/cdk binding site, such that said cell-proliferating disorder is treated; and

- b) monitoring the ability of said fusion protein to inhibit cell proliferation by measuring the light generated by said light-generating fusion protein.
30. A method of screening candidate compounds that inhibit cell proliferation, comprising the steps of:
- a) contacting cells with a candidate compound and a light-generating fusion protein comprising a cyclin/cdk binding site and a light-generating polypeptide moiety, wherein the light generation of said light-generating fusion protein changes upon binding of a cyclin at said cyclin binding site, said cyclin binding indicative of cancerous tissue; and
 - b) determining the ability of said candidate compound to inhibit cell proliferation by measuring the luminescence of said cells.
31. A cell proliferation-inhibiting compound identified by the method of claim 30.
32. A method for screening for a modulator of activity or latency of, or predisposition to a disorder, said method comprising:
- a) administering a test compound to a test animal at increased risk for a disorder, wherein said test animal recombinantly expresses a light-generating fusion protein comprising a ligand binding site and a light-generating polypeptide moiety, wherein the light generation of said light-generating fusion protein changes upon binding of a ligand at said ligand binding site, said ligand binding site recognizing a ligand on an entity associated with a disorder, or a product of said disorder;
 - b) allowing for localization of said light-generating fusion protein and an entity, wherein contact between said ligand binding site and a ligand associated with said disorder causes a modification of a colinear effector site which alters the light generation of said light-generating polypeptide moiety;
 - c) detecting the luminescence of said light-generating polypeptide moiety in said test animal after administering the compound of step (a); and
 - d) comparing the luminescence of said light-generating polypeptide moiety in said test animal with the luminescence of said light-generating polypeptide moiety in a control animal not administered said compound, wherein a change in the activity of said light-generating polypeptide moiety in said test

animal relative to said control animal indicates the test compound is a modulator of latency of or predisposition to, a disorder.

33. The method of claim 32, wherein the disorder is a hypoxia-related disorder.

34. The method of claim 32, wherein the disorder is selected from the group consisting of cancer, diabetes, heart disease and stroke.

35. A non-invasive method for determining the effects of an anti-hypoxic compound *in vivo*, comprising:

- a) administering to a subject a light-generating fusion protein, or a cell expressing said light-generating fusion protein, comprising an ubiquitin ligase binding site and a light-generating polypeptide moiety, wherein the light generation of said light-generating fusion protein changes upon binding of a ubiquitin ligase at said ubiquitin ligase binding site, said ubiquitin ligase binding site recognizing a ubiquitin ligase present in hypoxic conditions in hypoxic tissue;
- b) allowing for localization of said light-generating fusion protein or cell in hypoxic tissue in said subject, wherein contact between said ubiquitin ligase binding site and a ubiquitin ligase causes a modification of a colinear effector site which alters the light generation of said light-generating polypeptide moiety; and;
- c) determining the ability of said candidate compound to inhibit hypoxia by measuring the luminescence of said localized light-generating fusion protein.

36. The method of claim 35, wherein said ligand binding site comprises the amino acid sequence Y-X₁-Leu-X₂-Pro_h-X₃-X₄-X₅-X₆-Y', wherein

- a) Pro_h is hydroxylated proline;
- b) X₁, X₂, X₄, X₅, and X₆ are independently Gly, Ala, Val, Leu, Ile, Pro, Met, Phe, or Trp;
- c) X₃ is Ser, Thr, or Tyr; and
- d) Y and Y' are independently present or absent and, if present, independently comprise a peptide having from 1 to 600 amino acids.

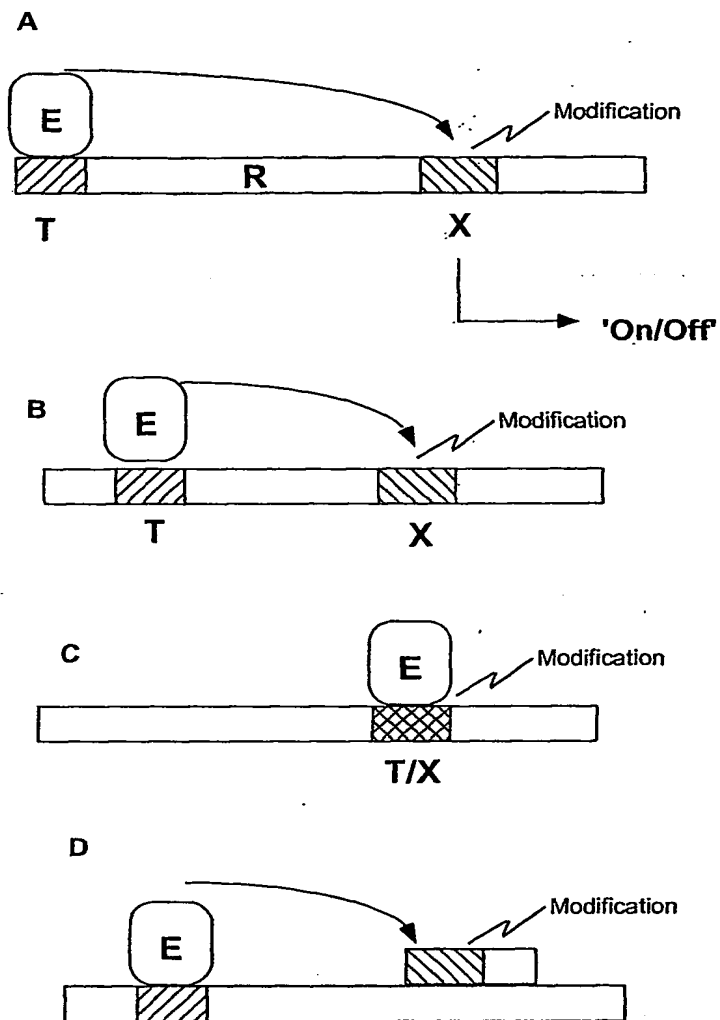
37. The method of claim 35, wherein said ligand binding site comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
38. The method of claim 35, wherein said ligand binding site comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 402 is hydroxylated proline.
39. The method of claim 35, wherein said ligand binding site comprises the amino acid sequence corresponding to the N-terminal residues 1-600 of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein either or both of residues 402 and 564 are hydroxylated proline.
40. The method of claim 35, wherein said ligand binding site comprises a 4 to 12 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
41. The method of claim 35, wherein said ligand binding site comprises a 12 to 14 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
42. The method of claim 35, wherein said ligand binding site comprises a 20 to 30 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
43. The method of claim 35, wherein said ligand binding site comprises a 80 to 120 amino acid sequence corresponding to the residues adjacent to and/or surrounding residue 564, inclusive, of HIF1 α , numbered in accordance with wild-type HIF1 α , wherein residue 564 is hydroxylated proline.
44. A noninvasive method for detecting the effects of an anti-cell proliferation compound under study in a mammalian subject, comprising:
 - a) administering to a subject a light-generating fusion protein, or a cell expressing said light-generating fusion protein, comprising a cyclin/cdk binding site and a light-generating polypeptide moiety, wherein the light

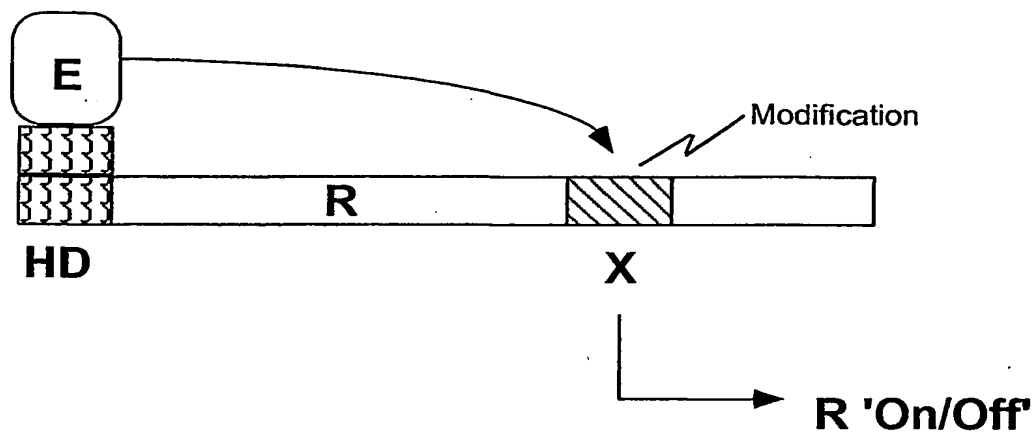
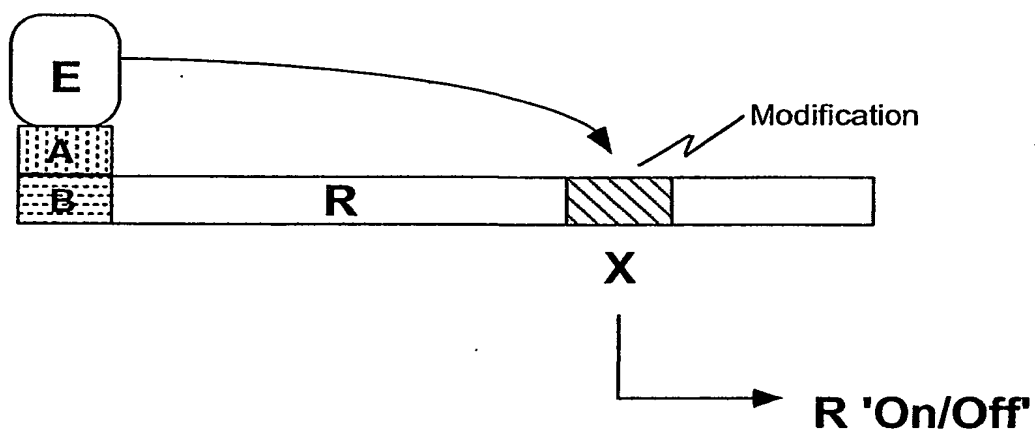
generation of said light-generating fusion protein changes upon binding of a cyclin at said cyclin binding site, said cyclin binding indicative of cell-proliferating tissue;

- b) allowing for localization of said light-generating fusion protein or cell in cell-proliferating tissue in said subject, wherein contact between said cyclin binding site and a cyclin causes a modification of a colinear effector site which alters the light generation of said light-generating polypeptide moiety; and
- c) determining the ability of said candidate compound to inhibit cell proliferation by measuring the luminescence from said localized light-generating fusion protein.

- 45. A method of treating or preventing a hypoxic or ischemic related disorder in a subject, comprising administering to a subject in need thereof a compound which modulates prolyl hydroxylation of HIF, such that said hypoxic or ischemic related disorder is treated.
- 46. The method of claim 46, wherein said compound decreases prolyl hydroxylation of HIF.
- 47. The method of claim 46, wherein said compound increases prolyl hydroxylation of HIF.
- 48. A method of regulating HIF turnover in a subject, comprising administering to a subject in need thereof a compound which modulates prolyl hydroxylation of HIF, such that HIF turnover is regulated.
- 49. The method of claim 49, wherein said compound increases prolyl hydroxylation of HIF.
- 50. The method of claim 49, wherein said compound decreases prolyl hydroxylation of HIF.
- 51. A method of treating or preventing a HIF-related disorder in a subject, comprising administering to a subject in need thereof a compound which modulates prolyl hydroxylation of HIF, such that said HIF-related disorder is prevented, reversed or stabilized.

52. The method of claim 52, wherein said compound increases prolyl hydroxylation of HIF.
53. The method of claim 52, wherein said compound decreases prolyl hydroxylation of HIF.
54. The method of claim 52, wherein said HIF-related disorder is selected from the group consisting of myocardial infarction, stroke, cancer, and diabetes.

**FIG 1**

A**B****FIG 2**

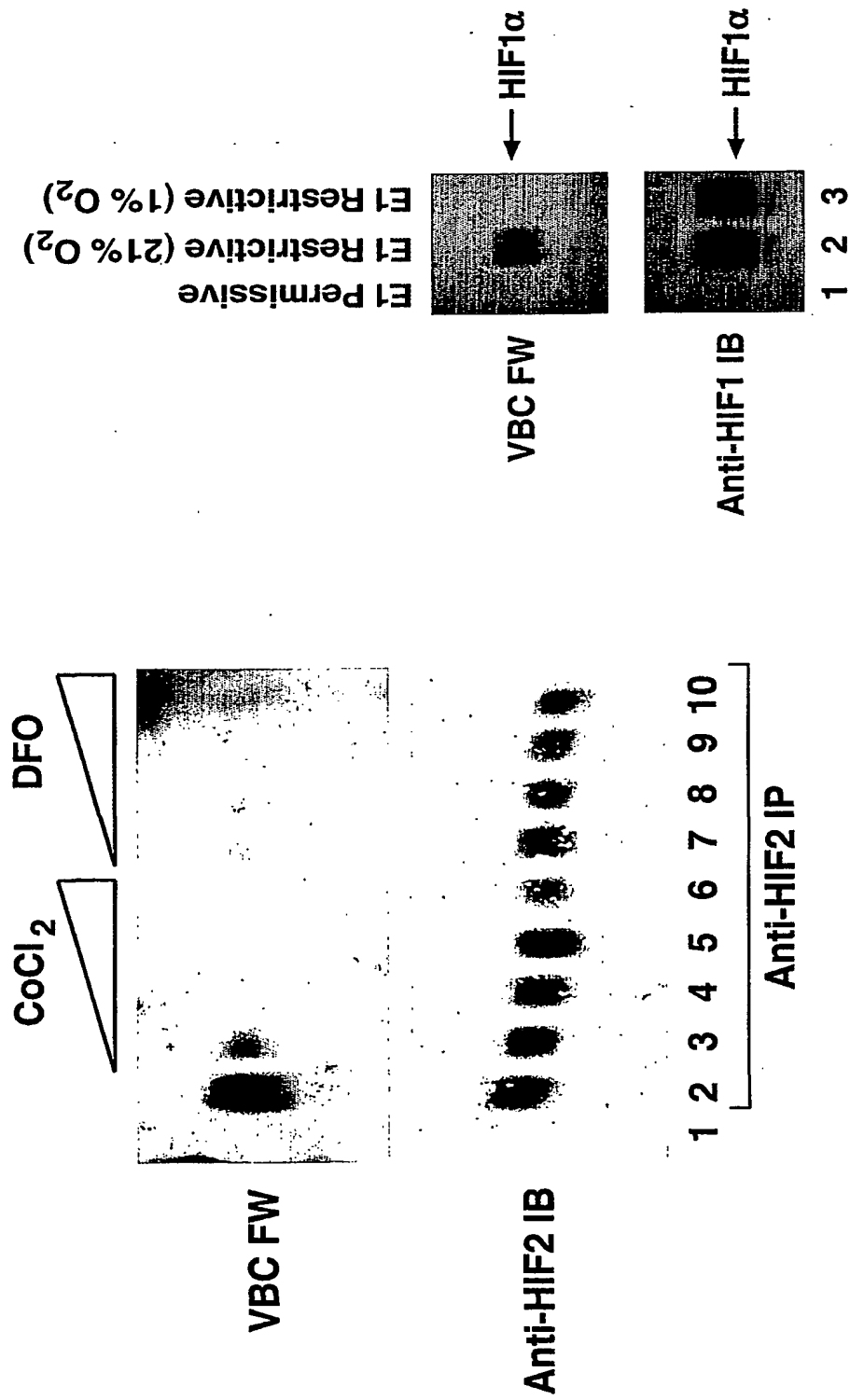


FIG 3B

FIG 3A

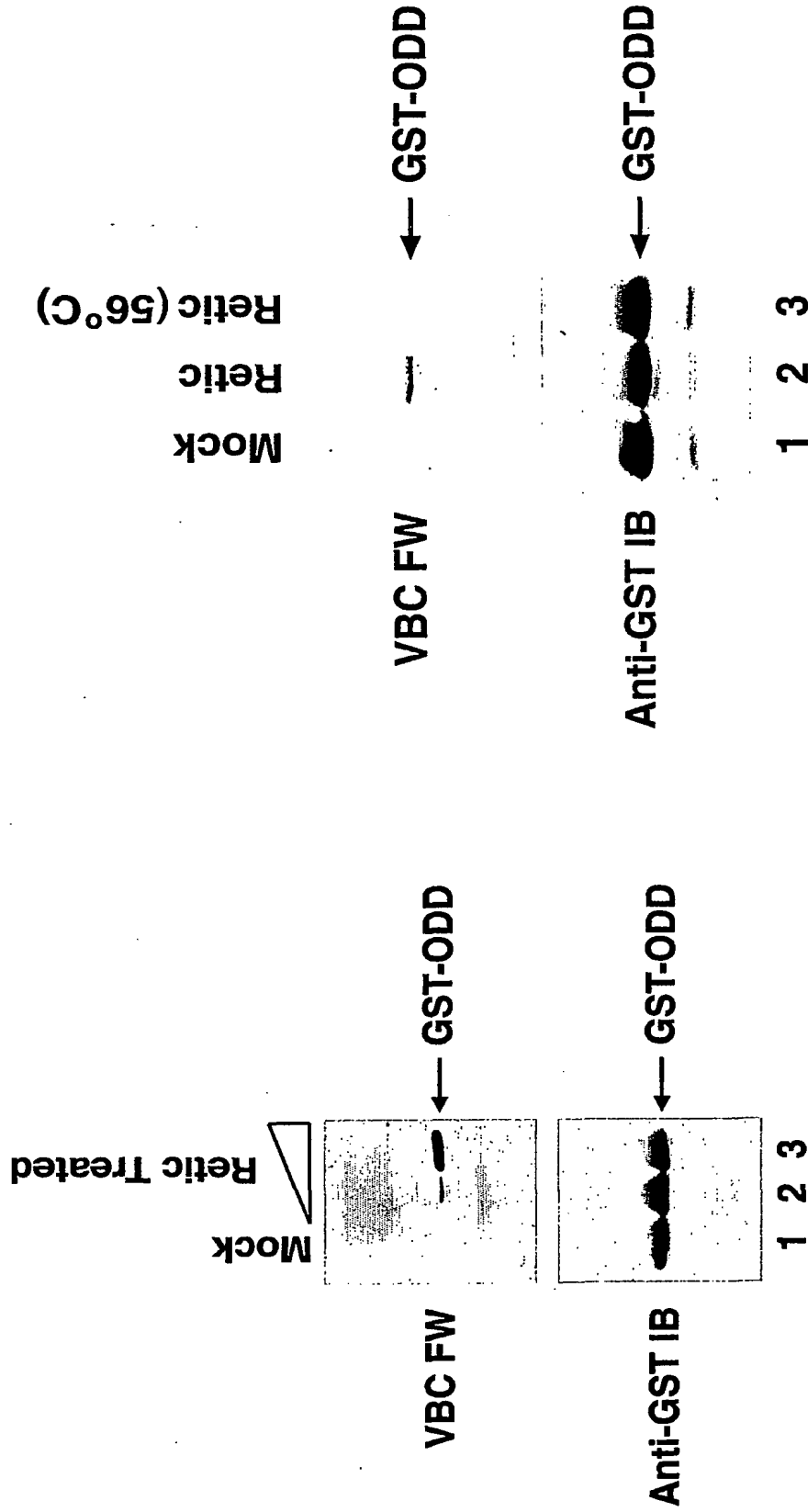


FIG 3D

FIG 3C

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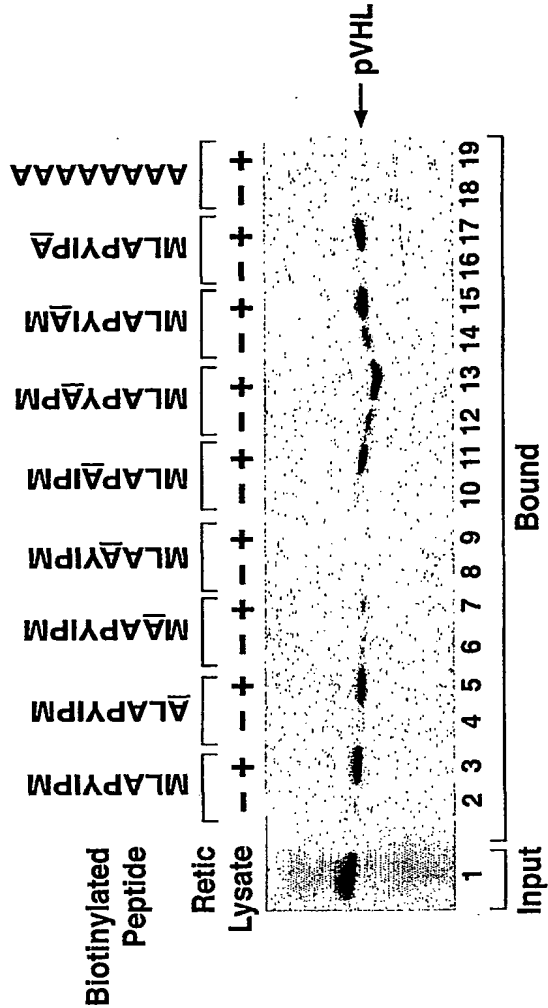


FIG 4B

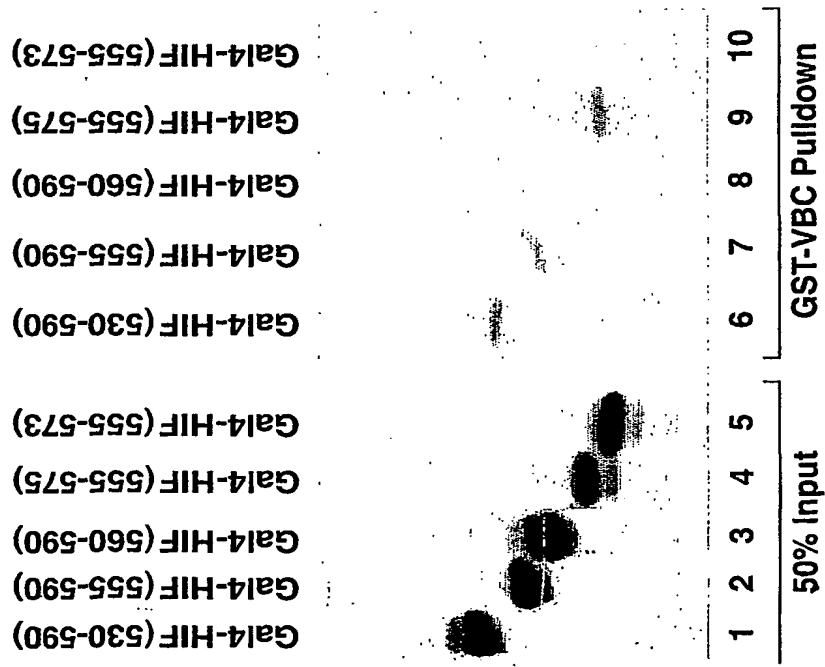


FIG 4A

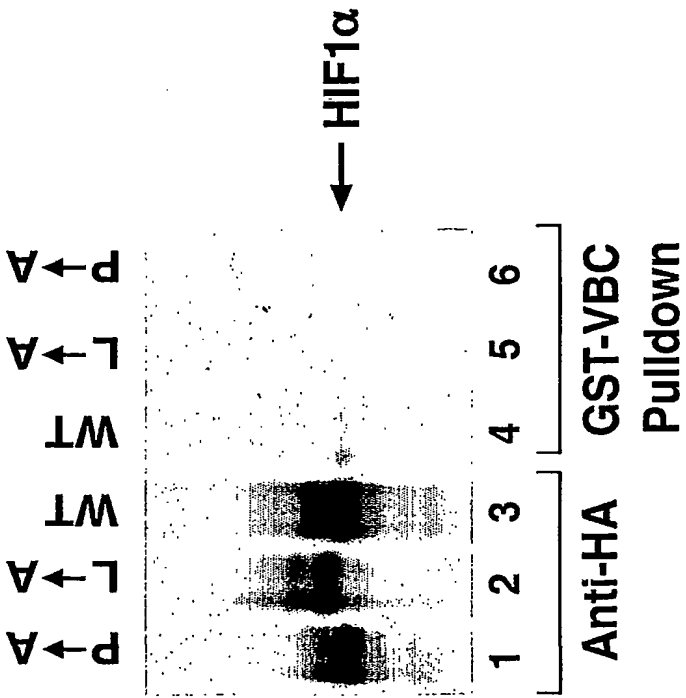


FIG 4C

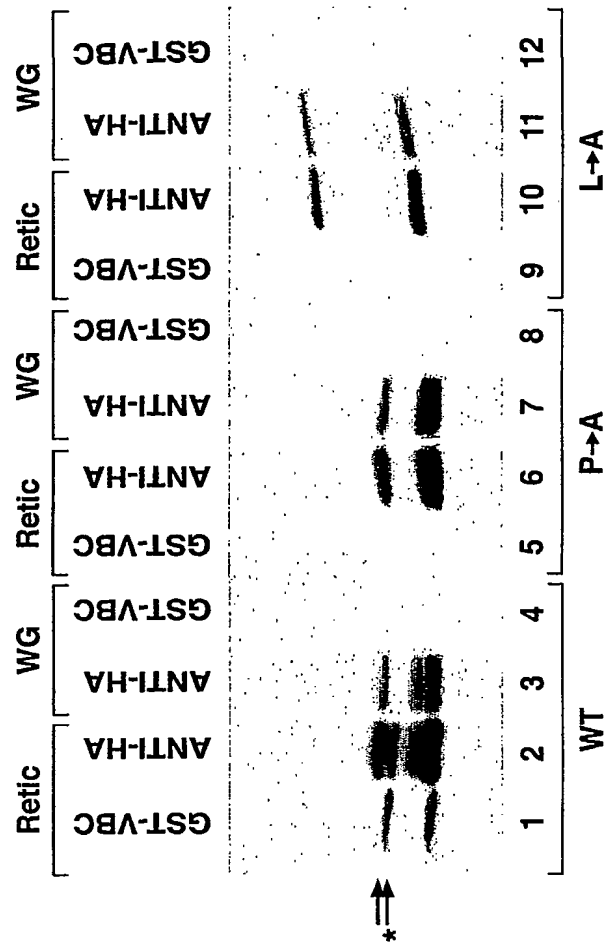


FIG 4D

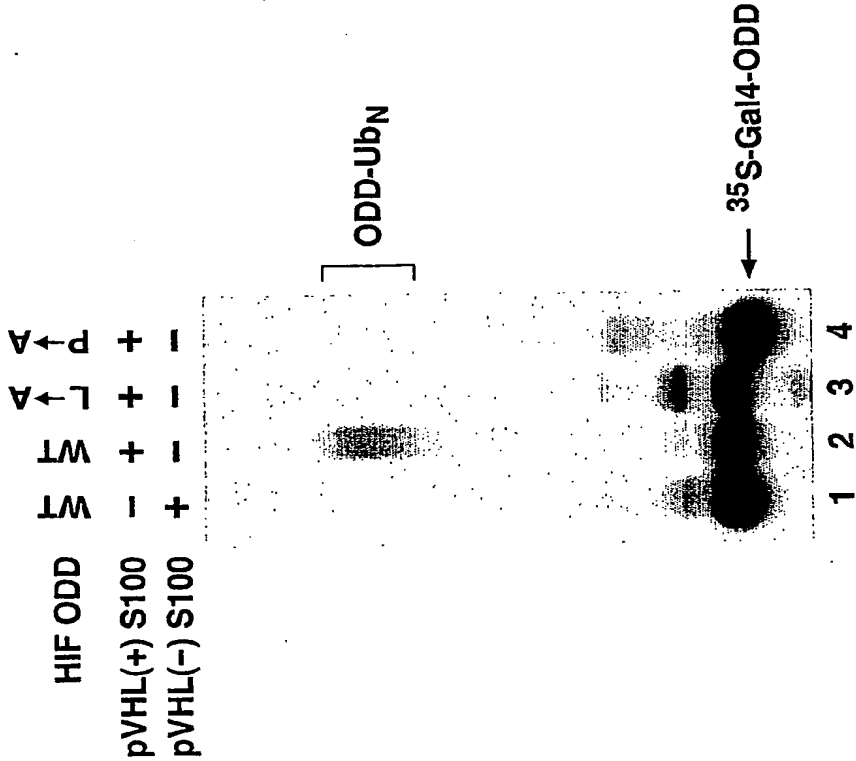


FIG 5A

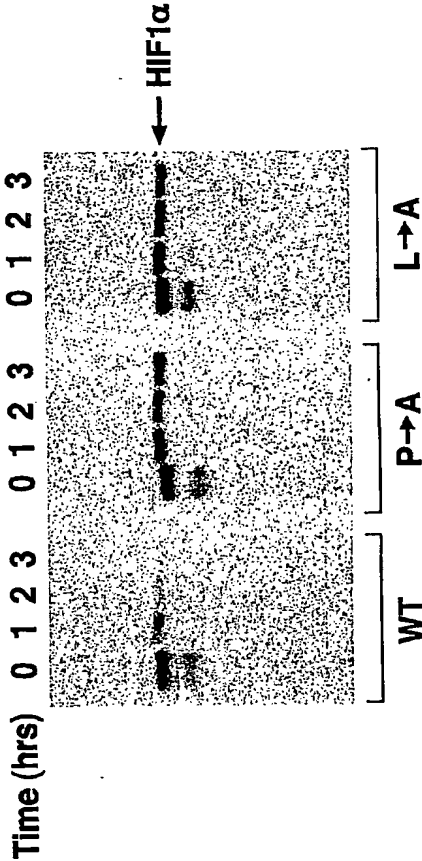


FIG 5B

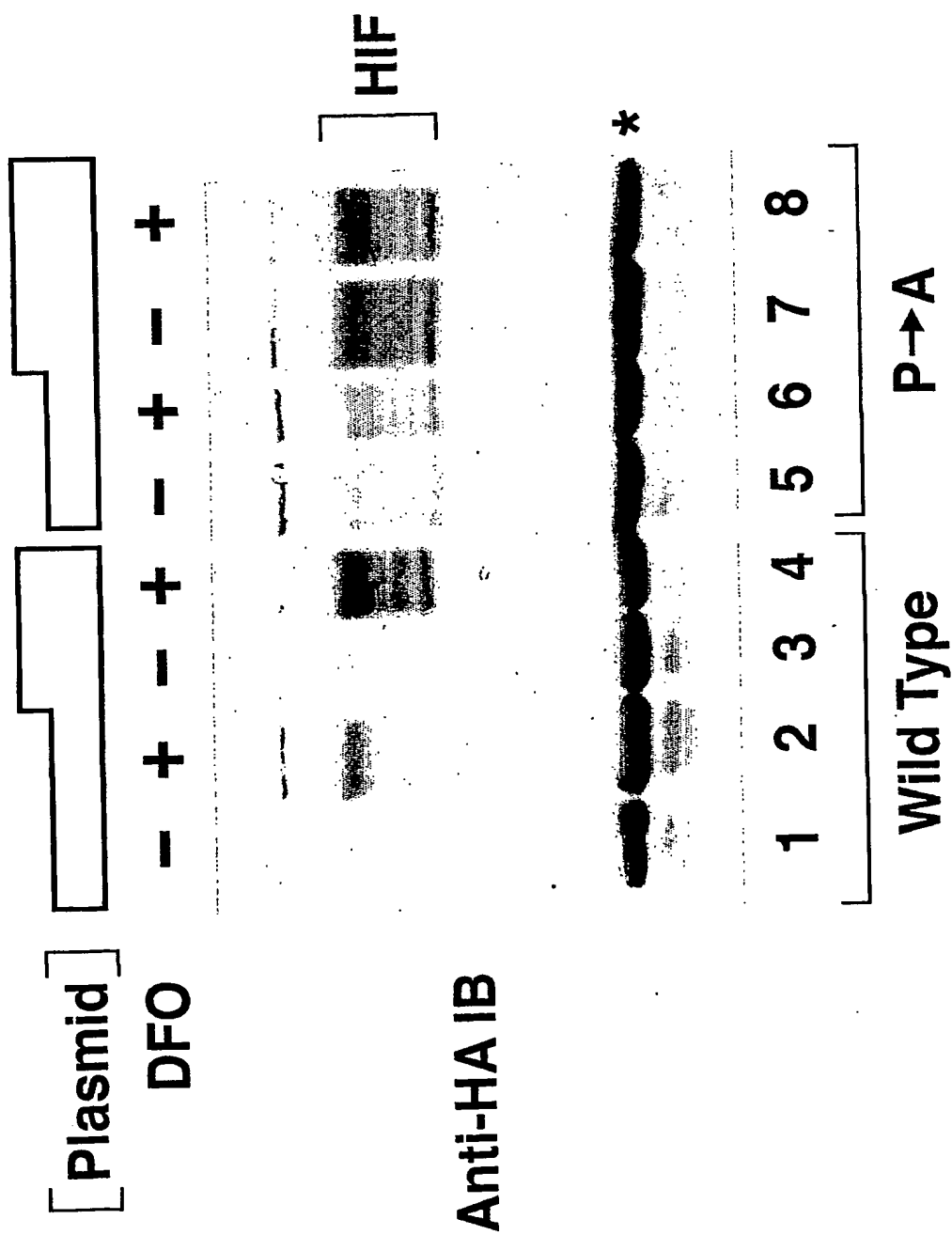
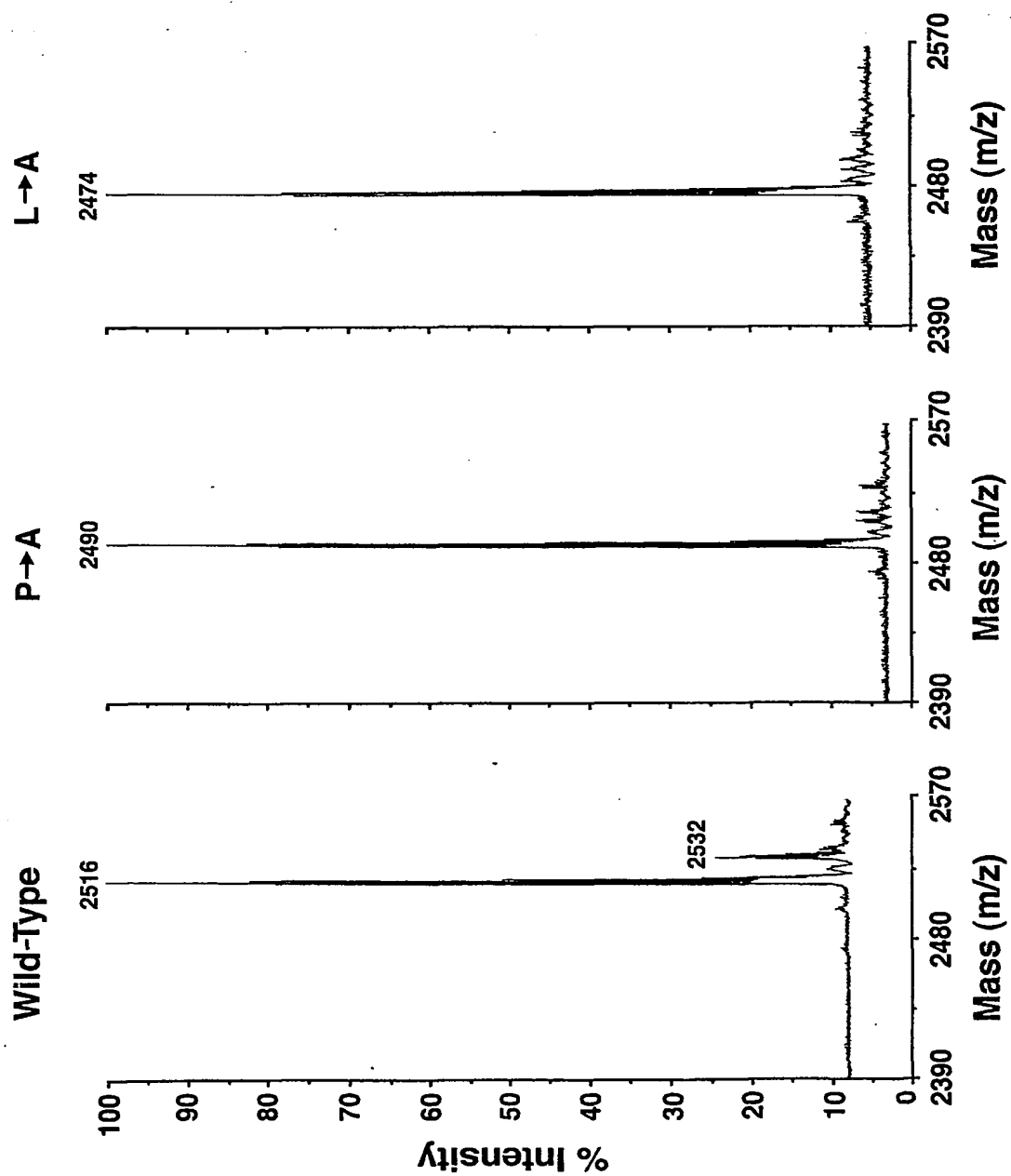


FIG 5C

**FIG 6A**

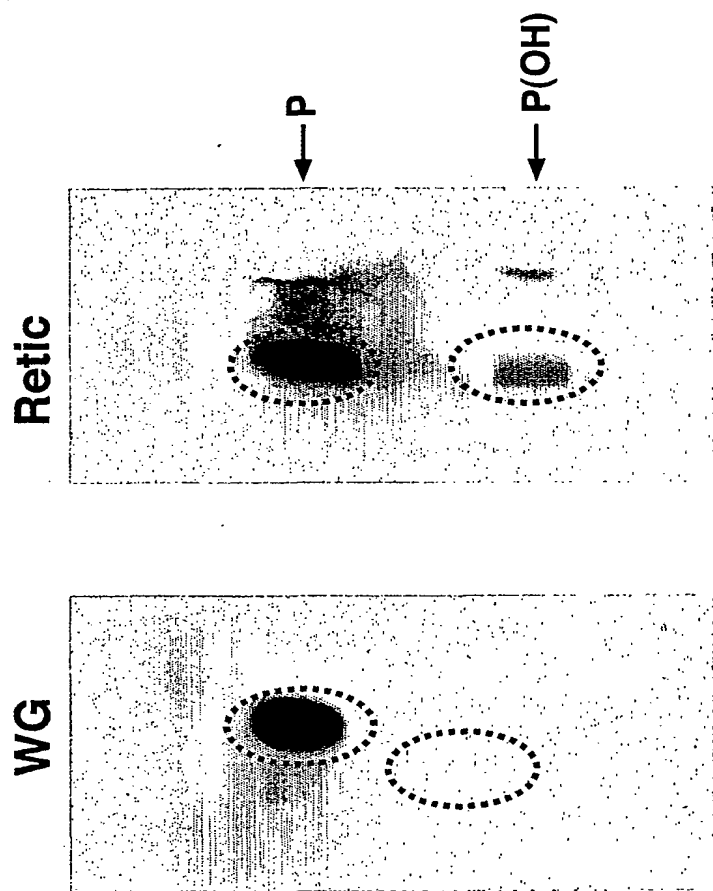


FIG 6B

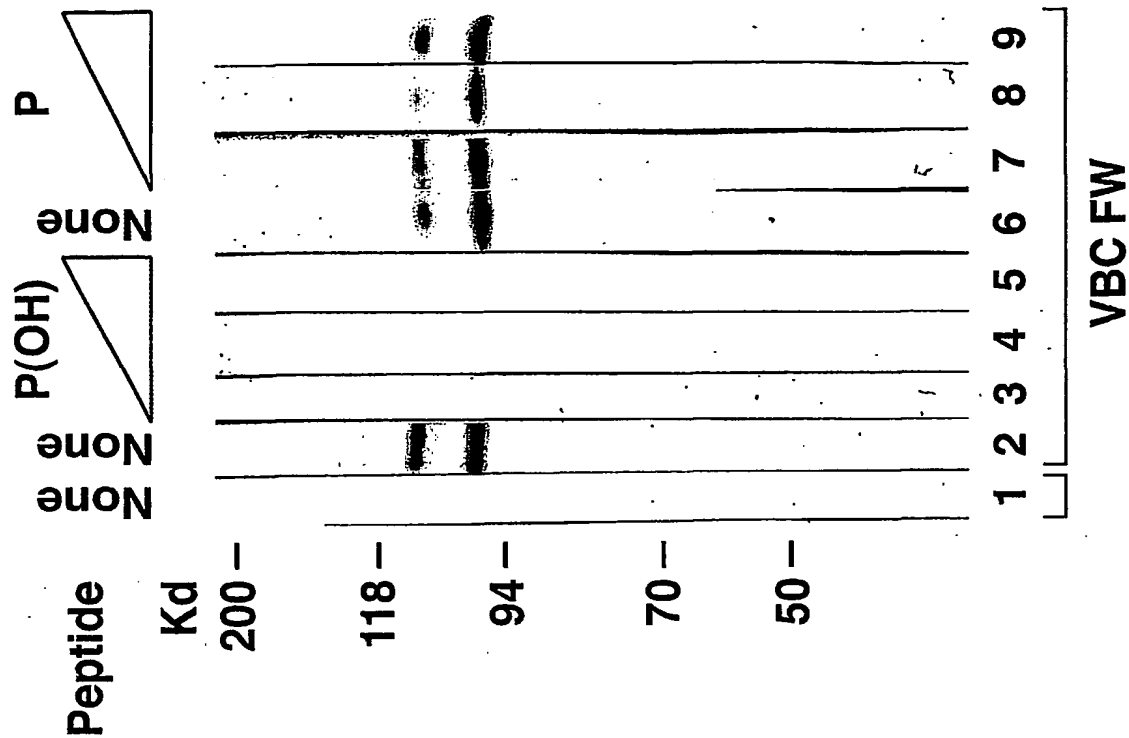


FIG 6D



FIG 6C

12/27

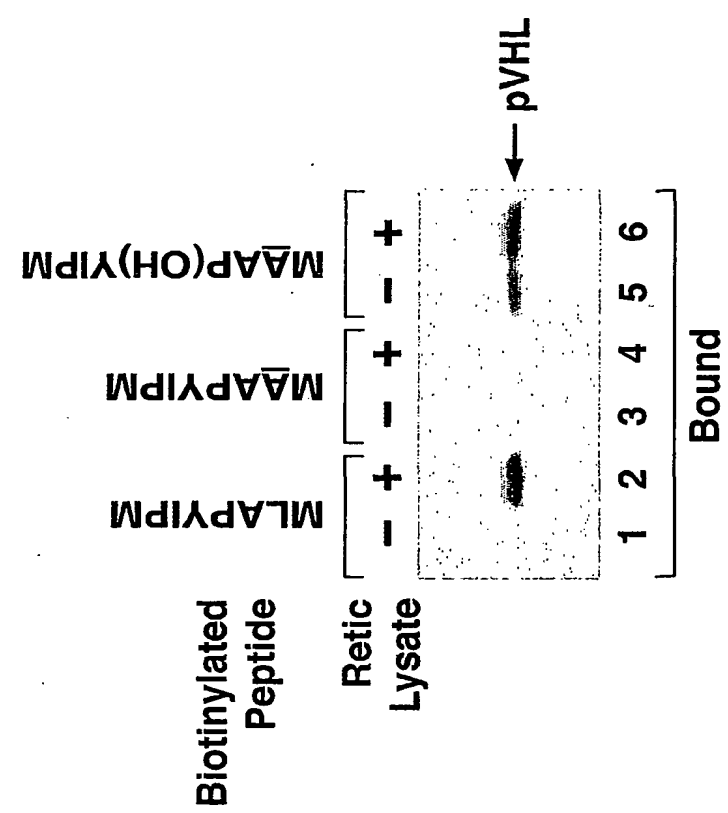


FIG 7B

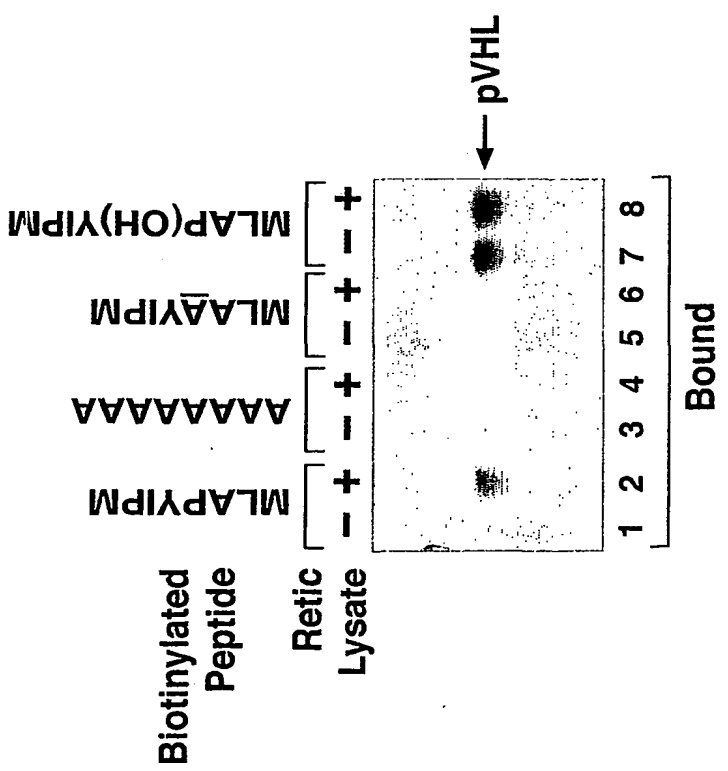


FIG 7A

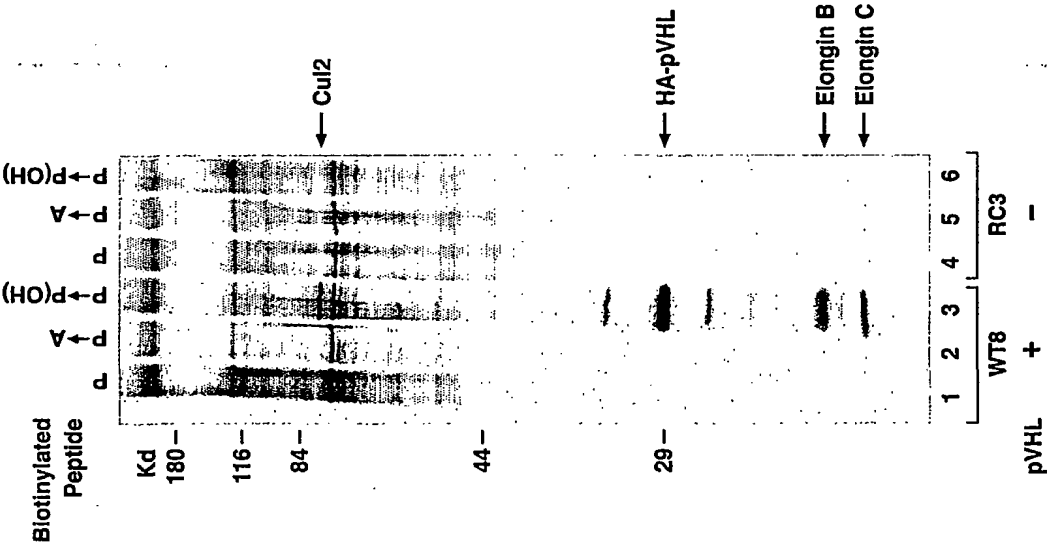


FIG 7D

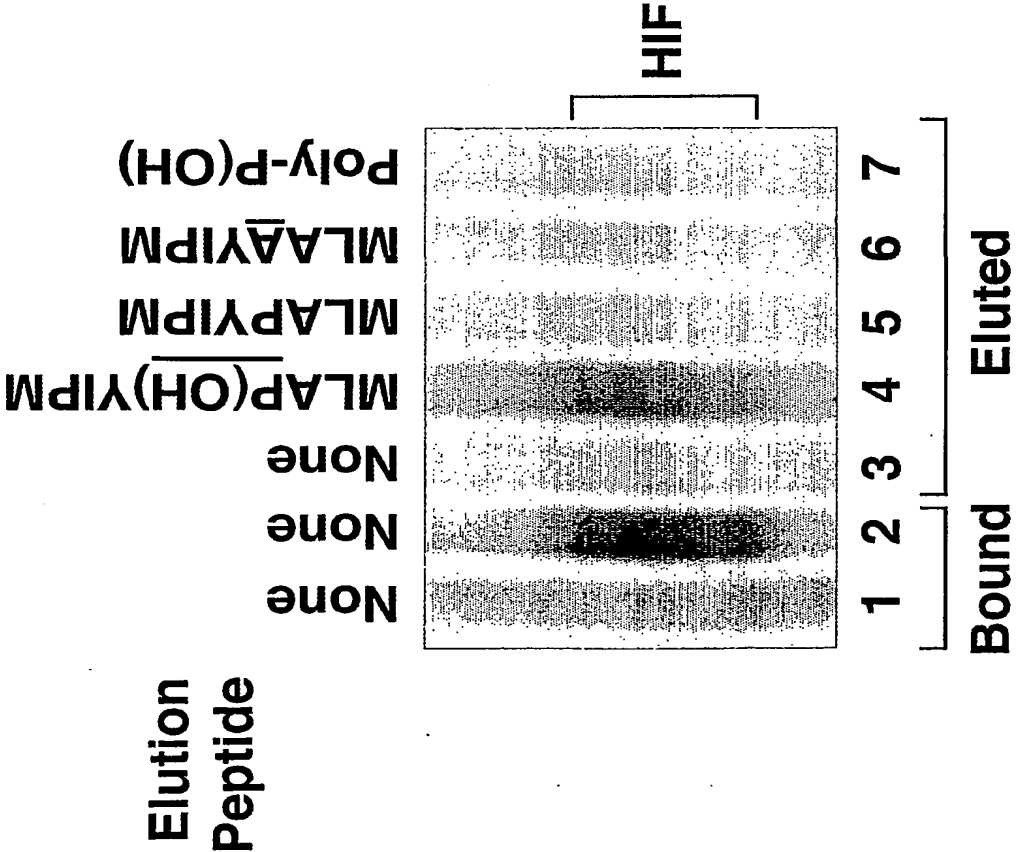


FIG 7C

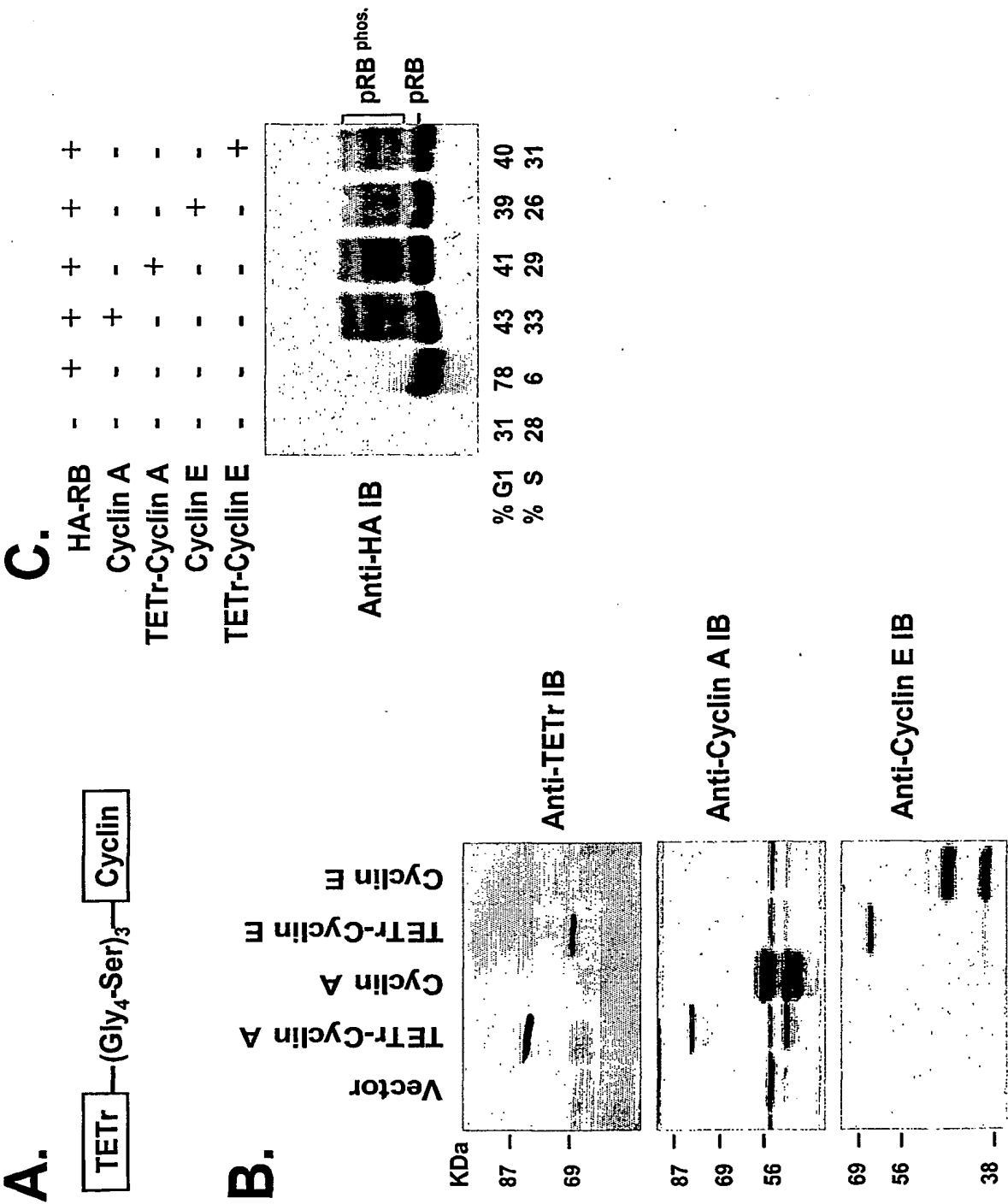
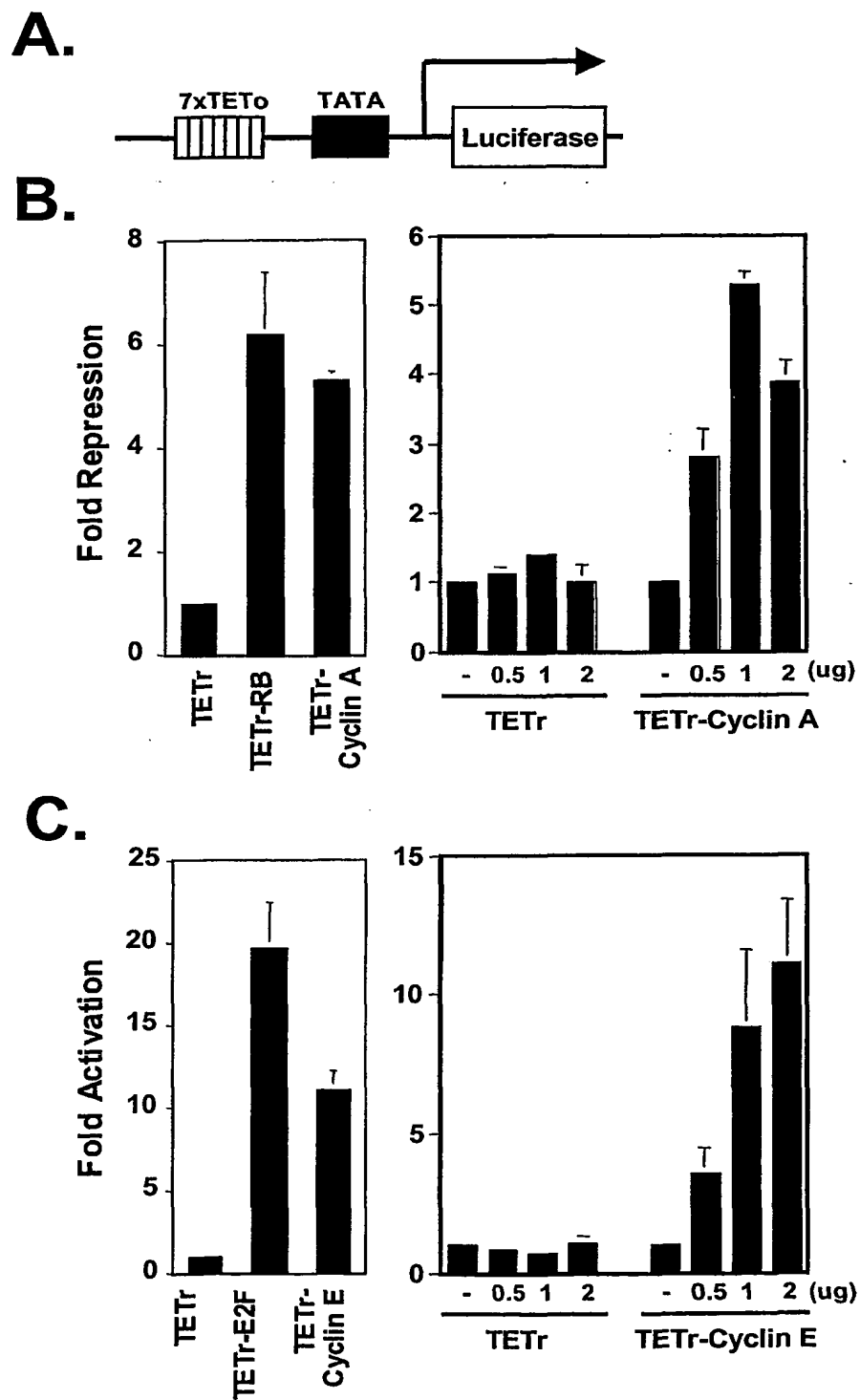


FIG. 8

**FIG. 9**

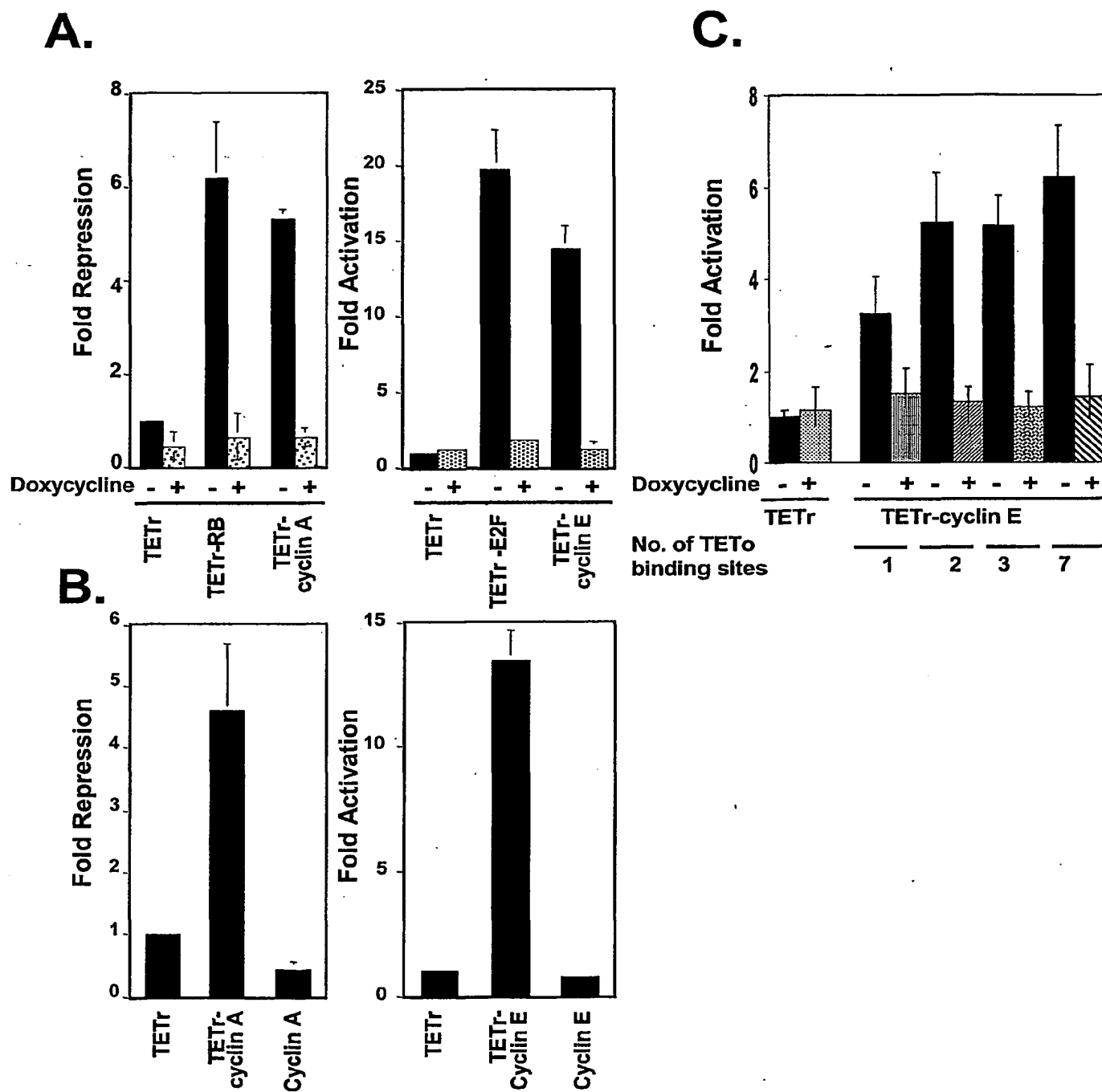


FIG. 10

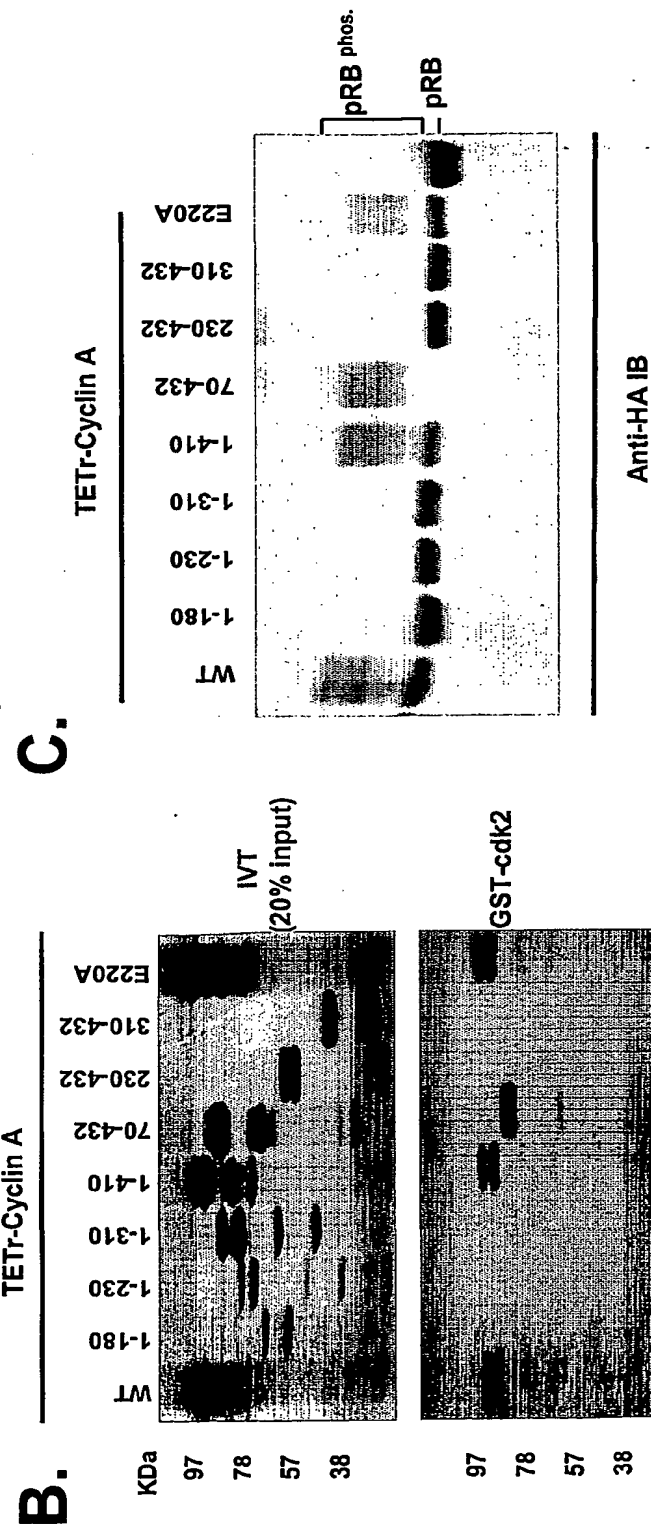
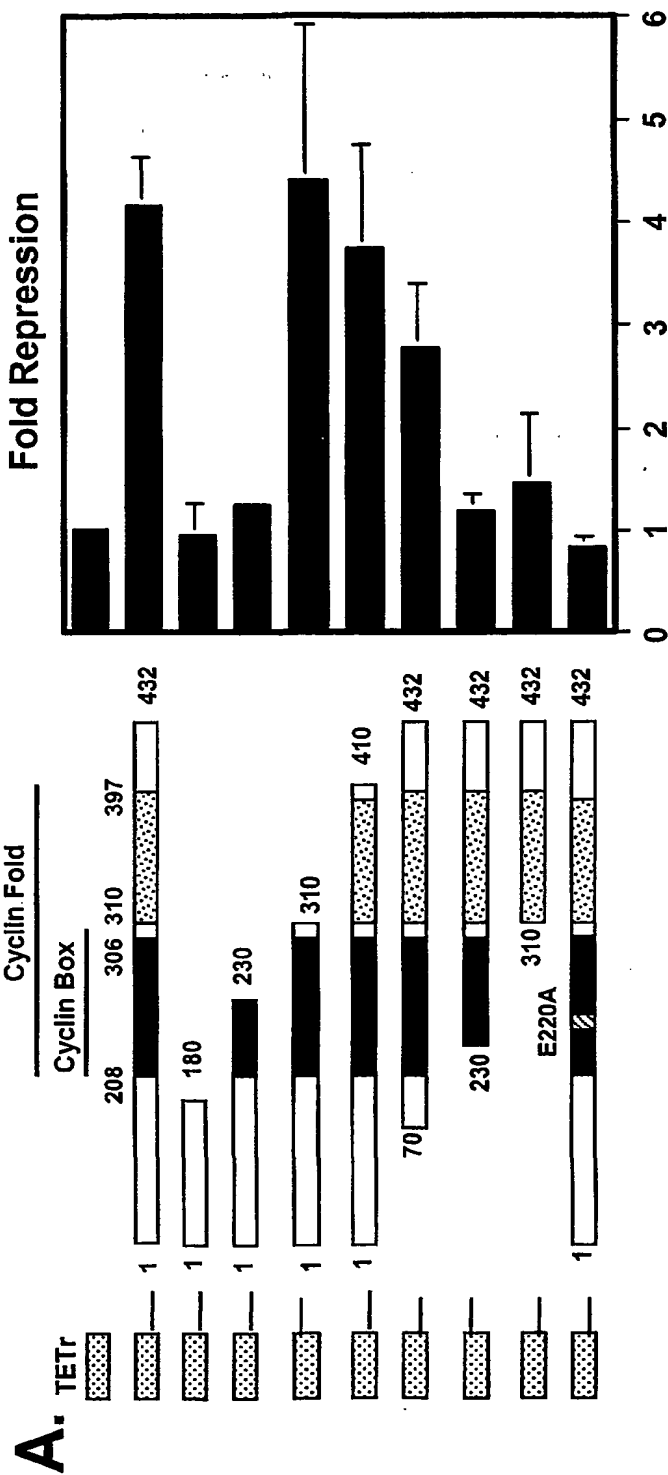


FIG. 11

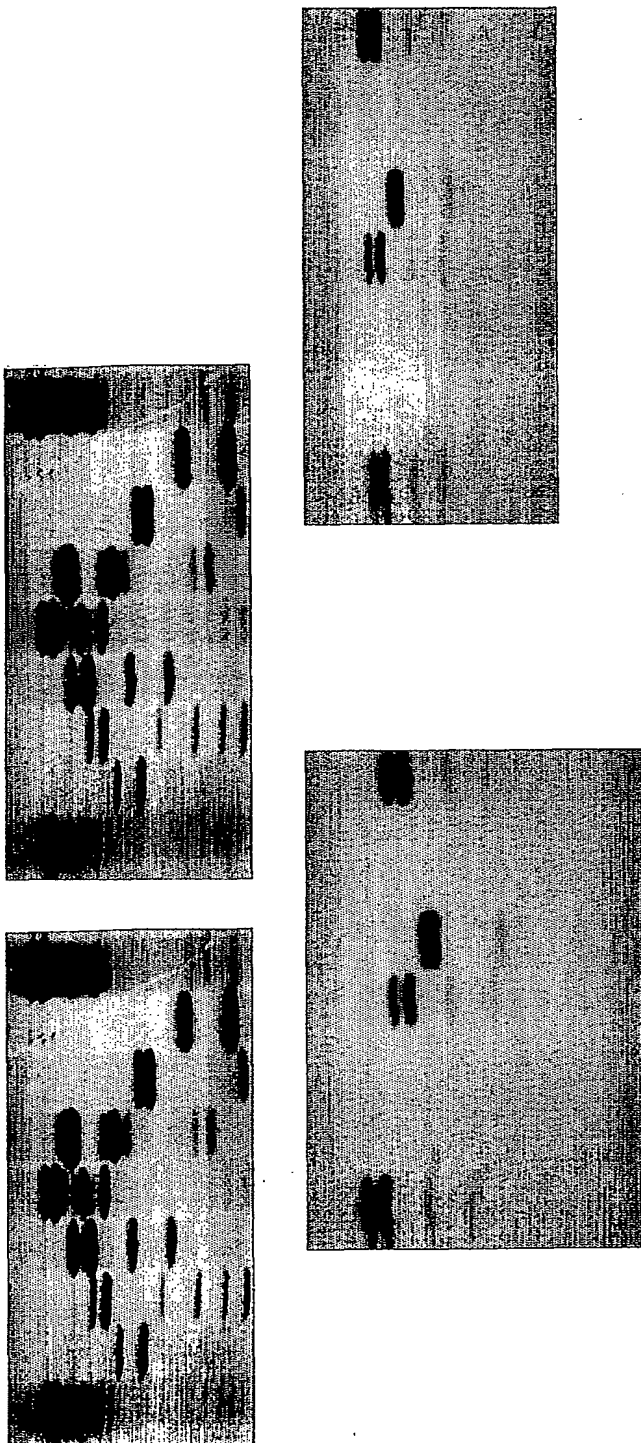


FIG. 11 (con't)

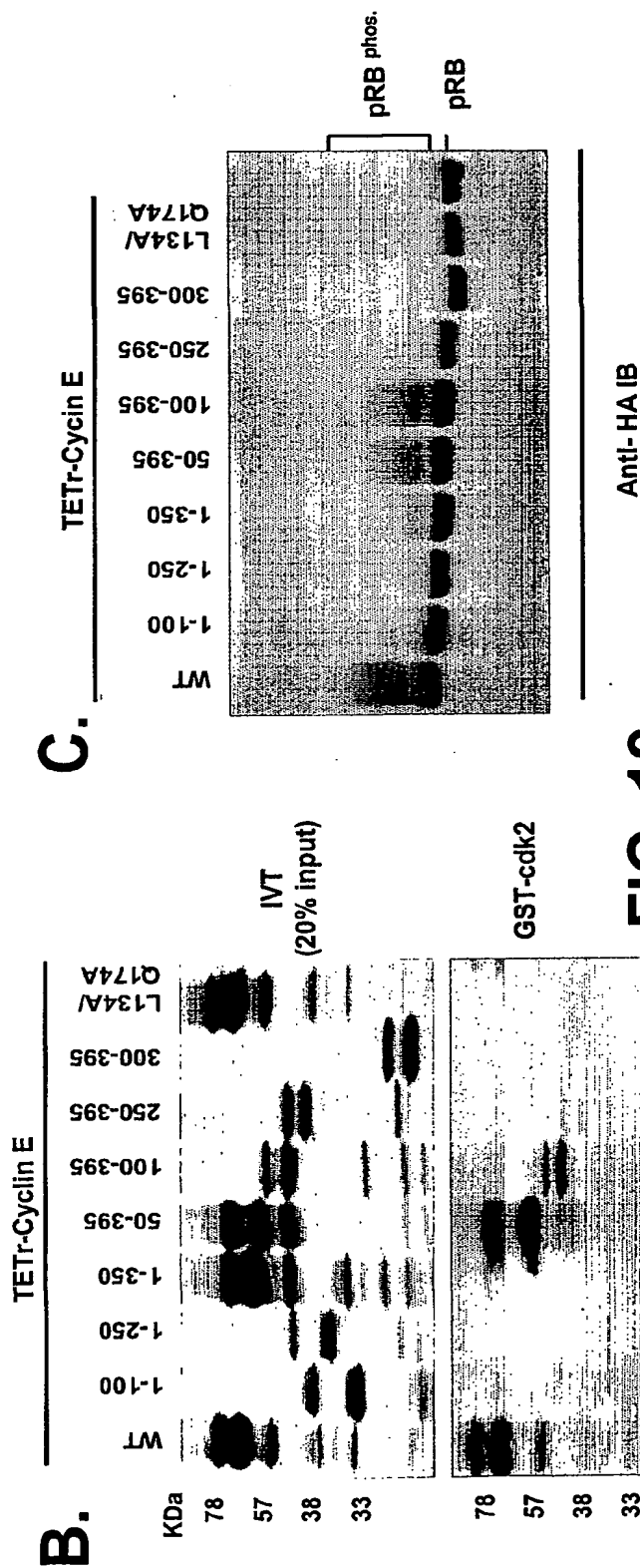
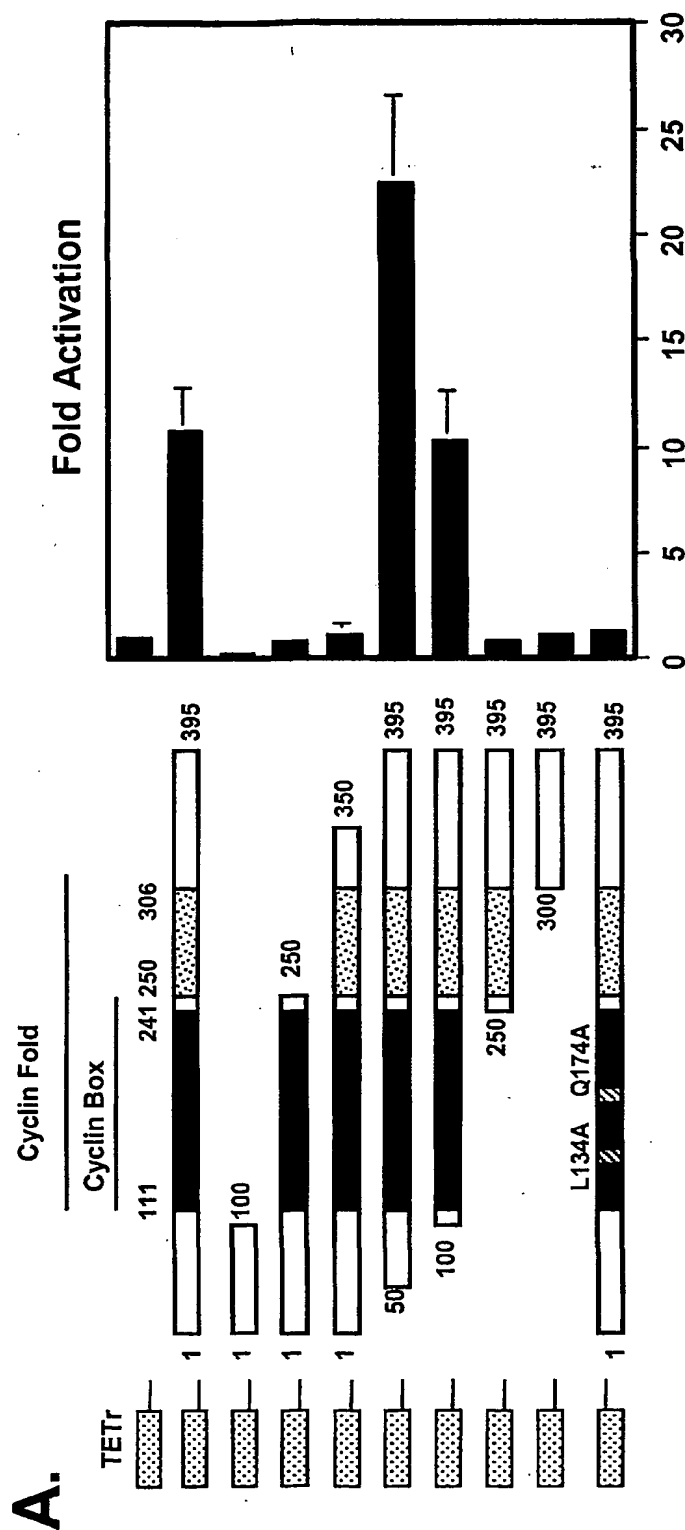
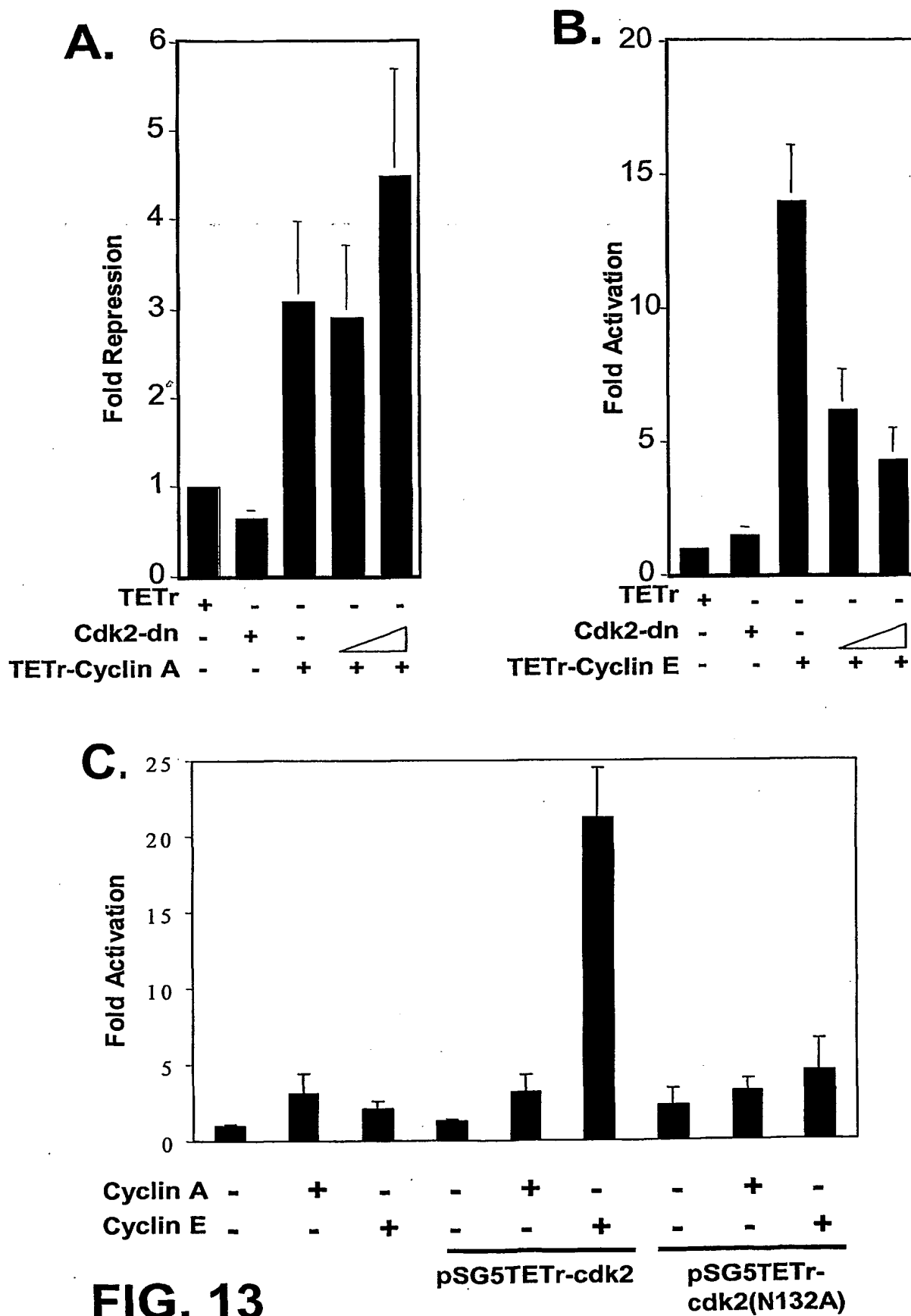


FIG. 12



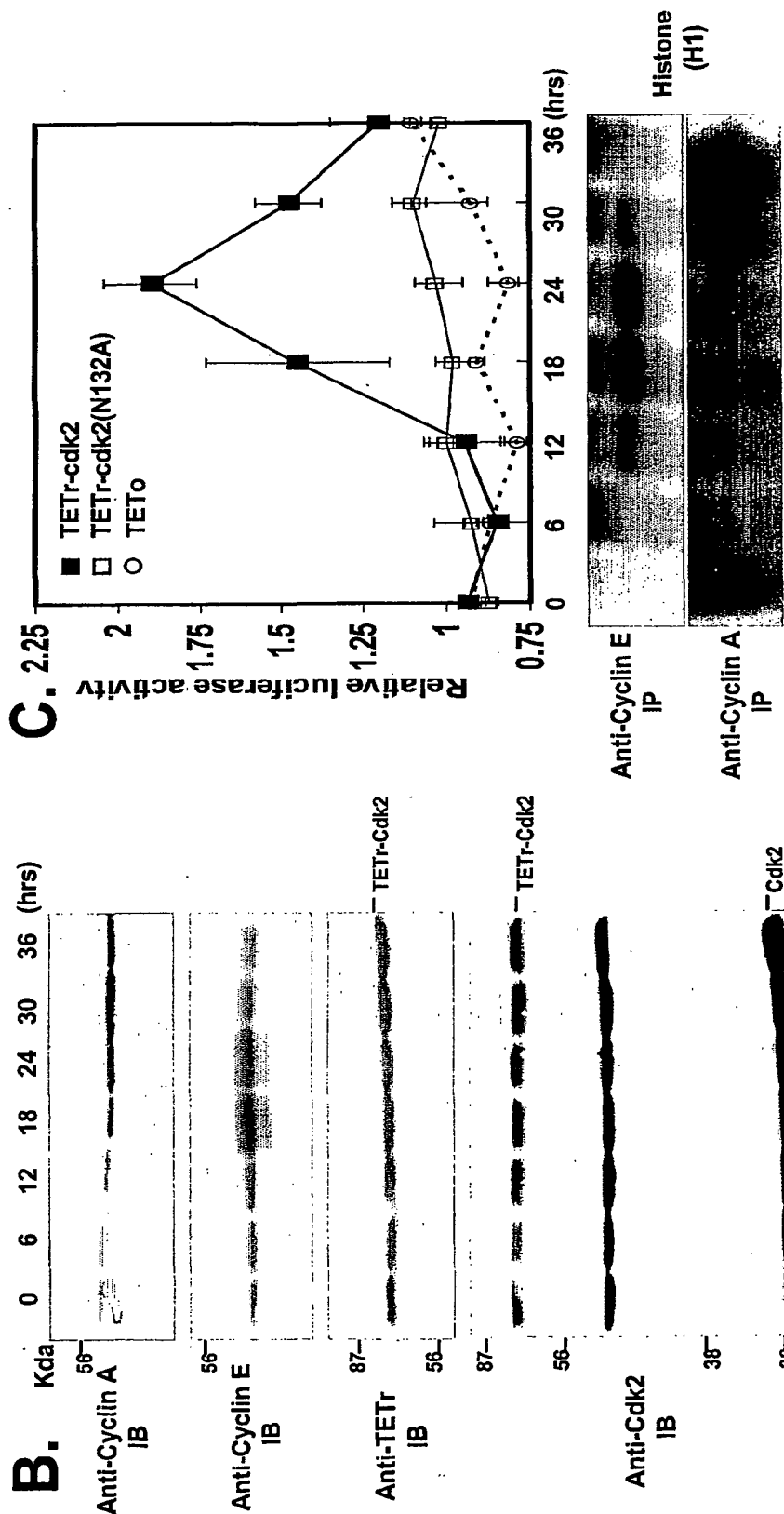
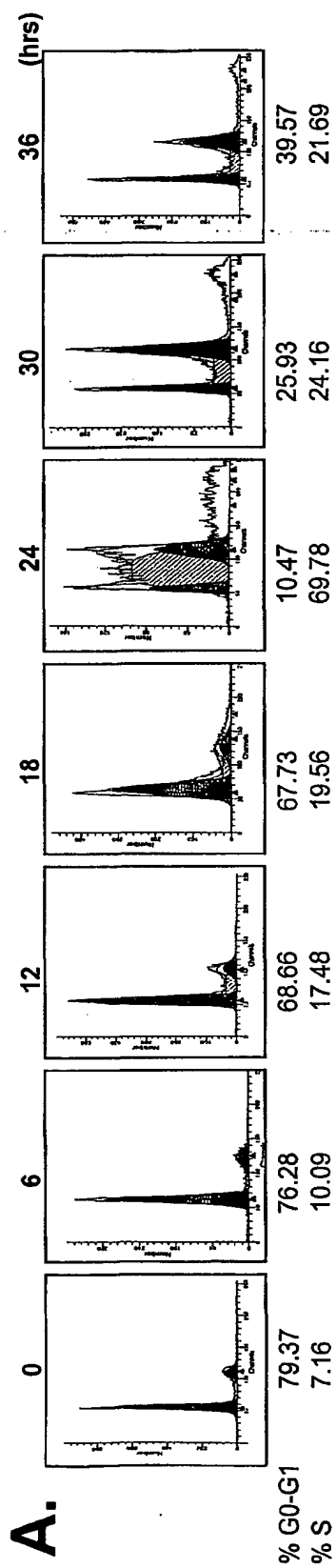
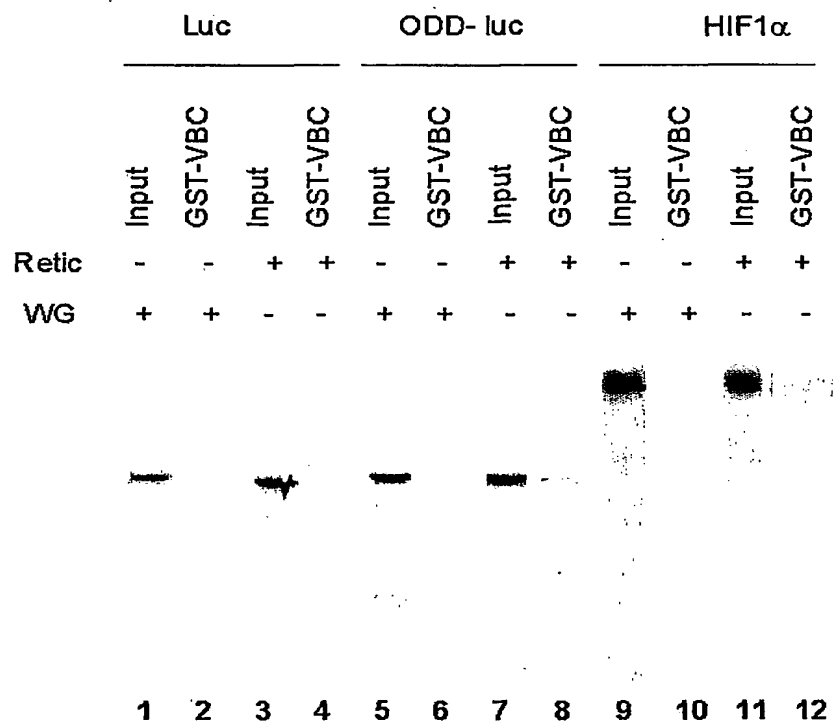
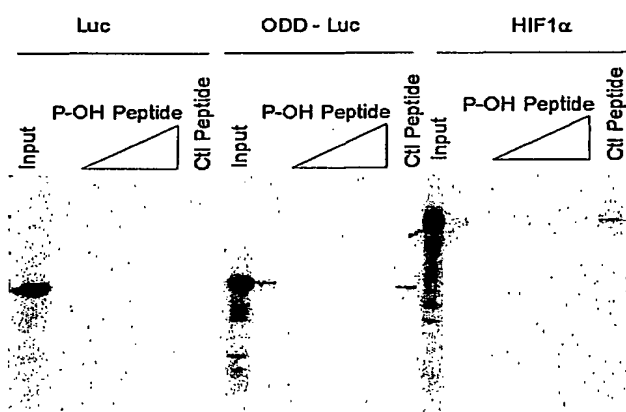
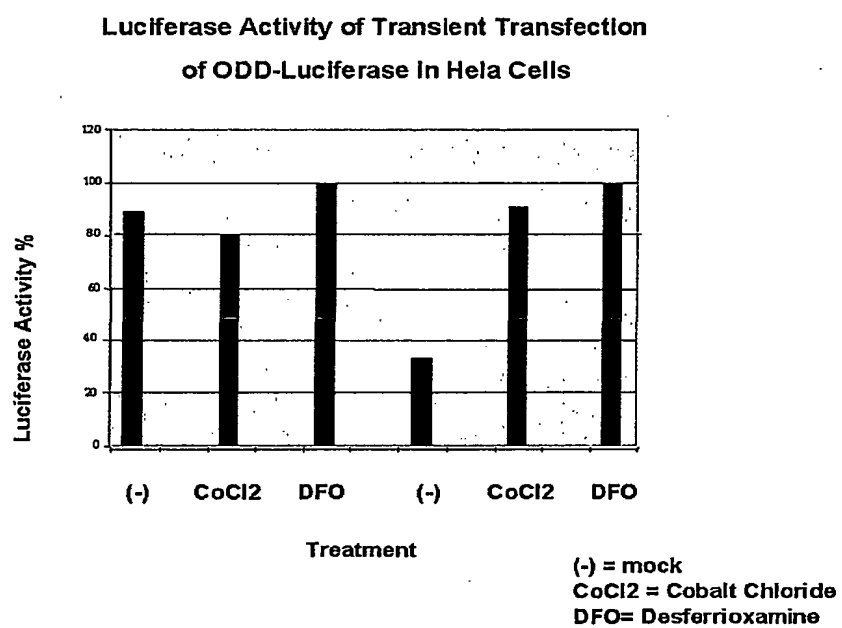


FIG. 14

GST - VBC Pulldown: Reticulocyte -VS- Wheat Germ**FIG 15**

P-OH Peptide Competition Assay**FIG 16**

**FIG 17**

Imaging of ODD-Luc in Xenograft Tumors

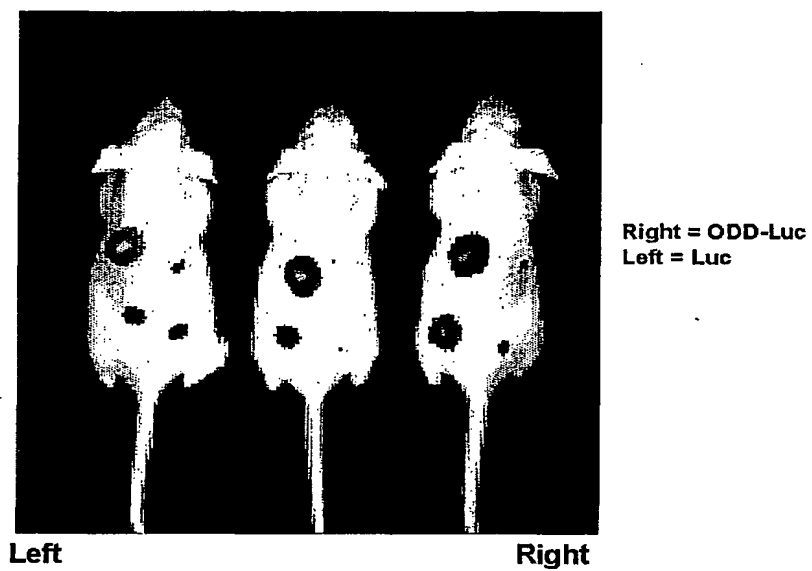
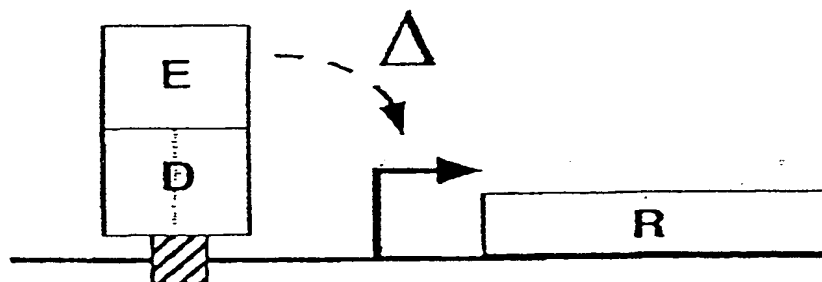


FIG 18

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FIG 19



D = DNA-Binding Domain e.g. TETr

E = Enzyme or Enzyme Partner e.g. Cdk2

R = Reporter cDNA e.g. Luciferase

▨ = Binding Site for 'D' e.g. TETo

└─▶ = Promoter

FIG 20

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Kaelin Jr., William G
Ivan, Mircea

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Screening Methods Thereof

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<223> A peptide of 1 to 600 amino acids wherein Xaa is any amino acid.

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35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

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<223> A peptide from 1 to 600 amino acids long wherein
Xaa is any amino acid.

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acids.

<220>
<221> VARIANT
<222> (605)
<223> Wherein Xaa is one or more of any of the amino
acids.

<220>
<221> VARIANT
<222> (607)..(1206)
<223> A peptide from 1 to 600 amino acids long wherein
Xaa is any amino acid.

<400> 9
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15
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20 25 30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Consensus
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 35 40 45
 Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
 50 55 60
 Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
 65 70 75 80
 Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
 85 90 95
 Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
 100 105 110
 Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
 115 120 125
 Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
 130 135 140
 Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
 145 150 155 160

Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
 165 170 175
 Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
 180 185 190
 His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
 195 200 205
 Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
 210 215 220
 Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
 225 230 235 240
 Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp
 245 250 255
 Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly
 260 265 270
 Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr
 275 280 285
 Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln
 290 295 300
 Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln
 305 310 315 320
 Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val
 325 330 335
 Cys Val Asn Tyr Val Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe
 340 345 350
 Ser Leu Gln Gln Thr Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp
 355 360 365
 Met Lys Met Thr Gln Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser
 370 375 380
 Ser Leu Phe Asp Lys Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu
 385 390 395 400
 Ala Pro Ala Ala Gly Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn
 405 410 415
 Asp Thr Glu Thr Asp Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn
 420 425 430
 Asp Val Met Leu Pro Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu
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 Ala Met Ser Pro Leu Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser
 450 455 460
 Ser Ala Asp Pro Ala Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro
 465 470 475 480
 Asn Pro Glu Ser Leu Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp
 485 490 495

Gln Thr Pro Ser Pro Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu
 500 505 510
 Pro Asn Ser Pro Ser Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val
 515 520 525
 Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr
 530 535 540
 Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu
 545 550 555 560
 Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser
 565 570 575
 Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser
 580 585 590
 Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln
 595 600 605
 Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu
 610 615 620
 Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala
 625 630 635 640
 Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser
 645 650 655
 Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala
 660 665 670
 Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro
 675 680 685
 Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu
 690 695 700
 Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg
 705 710 715 720
 Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr
 725 730 735
 Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp
 740 745 750
 Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln
 755 760 765
 Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly
 770 775 780
 Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys
 785 790 795 800
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 Glu Leu Leu Arg Ala Leu Asp Gln Val Asn
 820 825

<210> 11
 <211> 1208
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Consensus
 Binding Peptide

<220>
 <221> VARIANT
 <222> (1)..(600)
 <223> A peptide of 1 to 600 amino acids long wherein Xaa
 is any amino acid.

<220>
 <221> VARIANT
 <222> (601)
 <223> Wherein Xaa is Gly, Ala, Val, Leu, Ile, Pro, Met,
 Phe, or Trp.

<220>
 <221> VARIANT
 <222> (603)
 <223> Wherein Xaa is Gly, Ala, Val, Leu, Ile, Pro, Met,
 Phe, or Trp.

<220>
 <221> VARIANT
 <222> (605)
 <223> Wherein Xaa is Ser, Thr, or Tyr.

<220>
 <221> VARIANT
 <222> (606)..(608)
 <223> Wherein Xaa is Gly, Ala, Val, Leu, Ile, Pro, Met,
 Phe, or Trp.

<400> 11
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 1 5 10 15
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 20 25 30
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 35 40 45
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 50 55 60
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 85 90 95
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 100 105 110
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 115 120 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 130 135 140

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 145 150 155 160

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 165 170 175

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 180 185 190

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 195 200 205

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 210 215 220

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 225 230 235 240

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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995 1000 1005

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1205